High Efficiency Separation Techniques: Fast HPLC Using Monolithic Silica Columns and Chiral Separation Using Capillary Zone Electrophoresis

Von der Fakultät für Lebenswissenschaften

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von Sami Kamal Hamed Eldeeb

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1. Referent: Professor Dr. Hermann Wätzig
2. Referent: Professor Dr.Conrad Kunick
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Publikationen

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2- El Deeb, S., Schepers, U., Wätzig, H. A Fast HPLC method for the simultaneous determination of glimepiride, glibenclamide and related substances using monolithic columns. Journal of Separation Science 29: 1571-1577 (2006).

3- El Deeb, S., Wätzig, H. Performance comparison between monolithic C18 and conventional C18 particle-packed columns in the liquid chromatographic determination of propranolol HCl. Turkish Journal of Chremistry 30: 543-552 (2006).

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5- El Deeb, S., Preu, L., Wätzig, H. Neue Trends von der HPLC 2006. Pharmazie in unserer Zeit 6(35): 560-560 (2006).

6- El Deeb, S., Wätzig, H. Monolithic silca HPLC columns for the fast Liquid Chromatography. The Column 10: 12-14 (2006). www.the column.eu.com

7- Schepers, U., El Deeb, S., Ermer, J., Wätzig, H. Comparison of the recovery spread in analytical development and routine quality control-based on the ICH quality guideline Q2B. Journal of Pharmaceutical and Biomedical Analysis 43: 708-710 (2007).

8- El Deeb, S., Preu, L., Wätzig, H. Evaluation of monolithic HPLC columns: conventional method transfer, chromatographic parameters and batch to batch repeatability. Journal of Pharmaceutical and Biomedical Analysis 26: 62271-11 (2007) in press.

9- El Deeb, S., Preu, L., Wätzig, H. Strategies for the development of fast RP-HPLC methods using monolithic columns. Accepted by Journal of Separation Science (manuscript No. JSSC-2007-00092).

Tagungsbeiträge

1- El Deeb, S., Wätzig, H. Monolithic columns as an alternative approach to conventional columns for fast HPLC analysis. (Oral presentation 7) Analytical course of the Society of Germany Chemists at Institute of Pharmaceutical Chemistry, Technical University Braunschweig, September 21-23, (2005).

2- El Deeb, S., Wätzig, H. Strategy for developing reversed phase HPLC methods using monolithic silica columns. (Oral presentation V3B) The Annual Conference of the Association of Germany Pharmacists (DPhG-Doktorandentagung), Nürnberg-Heroldsberg, Germany, September 6-8, (2006).

3- El Deeb, S., Schepers, U., Wätzig, H. Evaluation of monolithic performance C18 HPLC columns in the rapid analysis of pilocarpine hydrochloride in the presence of its degradation products. (Poster C28) The Annual Conference of the Association of Germany Pharmacists (DPhG), Mainz, October 5-8, (2005).

4- El Deeb, S., Wätzig, H. A Fast HPLC method for the simultaneous determination of glimepiride, glibenclamide and related substances using monolithic columns. (Poster P-004-T) The 30th International Symposium and Exhibit on High Performance Liquid Phase Separation and Related Techniques (HPLC-2006), San Francisco, USA June 17-22, (2006).

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Dedication

To:

My Parents, Wife, Kids,

Sisters and Brother

List of Abbreviations

AF	asymmetry factor
AUC	area under curve
bar	atmosphere = 10^5 pascal (Pa)
BGE	background electrolyte
CD	cyclodextrin
CE	capillary electrophoresis
CGE	capillary gel electrophoresis
CIEF	capillary isoelectric focusing
cm	centimeter = 10^{-2} m
CM-β-CD	carboxymethylated-β-cyclodextrin
CZE	capillary zone electrophoresis
DAD	diode-array detector
DNS	dansyl
d_p	particle diameter
EOF	electro-osmotic flow
F	flow rate
FSCE	free solution capillary electrophoresis
HETP	height equivalent to theoretical plate
HP-β-CD	hydroxypropyl-β-cyclodextrin
HPLC	high performance liquid chromatography
HS-β-CD	high sulphated-β-cyclodextrin
ICH	international conference on harmonisation
I.D.	internal diameter of the column
LC	liquid chromatography
LOD	limit of detection
LOQ	limit of quantitation
m	meter
µ	micro = 10^{-6} meter
MEKC	miceller electrokinetic capillary chromatography
μ_{ep}	electrophoretic mobility
min	minute

m*M*	millimolar = 10^{-3} Molar
nl	nanoliter = 10^{-9} l
PEEK	poly(ether ethyl ketone)
pK_a	ionization constant
RP	reversed-phase
RP-HPLC	reversed-phase high performance liquid chromatography
RPC	reversed phase chromatography
RSD	relative standard deviation
s	second
SFC	supercritical-fluid chromatography
S/N ratio	signal-to-noise ratio
UPLC	ultra performance liquid chromatography
UV/Vis	ultraviolet/visible
V	volt
v/v	volume-by-volume

List of Symbols

α	directly proportional
H	plate height
k	retention factor ($k = t_R\text{-}t_o/t_o$)
L	length
L_d	length of capillary to the detector
L_t	total capillary length
N	plate number
r	radius
R_s	resolution value
R^2	coefficient of determination
T	absolute temperature
t	time
t_o	time zero, elution time of non-retained solute
t_m	migration time
t_R	retention time
w	peak width at base
$w_{1/2}$	peak width at half-height

1 Introduction

1.1 High Performance Liquid Chromatography (HPLC)

1.1.1 Introduction to HPLC

High performance liquid chromatography (HPLC) has become one of the most important and fastest growing techniques in analytical laboratory. The basic theory behind high performance liquid chromatography is not new, but it was not until around 1969 that HPLC was developed in its present form. This development introduced the effective use of a small diameter packing material and columns, which allowed the chromatographer to perform separations faster and with greater resolution than had previously been attainable. The basic of separation in HPLC involves partitioning of the analyte molecule between the liquid mobile phase and the solid stationary phase [1].

1.1.2 HPLC components

The essential components of a complete HPLC system are solvent delivery system (pump), detector, fixed volume injector loop or autosampler, solvent reservoirs, packed column, data system and recorder. A schematic of a simplified HPLC system is shown in Figure 1.

1.1.2.1 Column

The column is probably the heart of HPLC system. The development of this column technology leads to the evolution of the HPLC instrumentation systems used today. The conventionally used HPLC columns are particle packed columns. The key of column selection when previous separation is not available resides in knowing the chemistry of the sample. Columns should never be dry. A dry column will eventually have voids because the packing will shrink away from the wall, which would result in band broadening. Before running a sample in HPLC the column should be equilibrated. Usually column equilibrium is achieved after passage of 10 – 20 column volumes of the new mobile phase through the column. Insufficient column equilibrium usually leads to retention difference [2].

1.1.2.2 Pump

The solvent delivery system or as it is commonly called the pump includes two major types, constant volume or flow and constant pressure. Constant volume pumps are mechanically driven systems, most commonly using screw driven syringes or

reciprocating pistons. On the other hand, constant pressure pumps are driven or controlled by gas pressure [3].

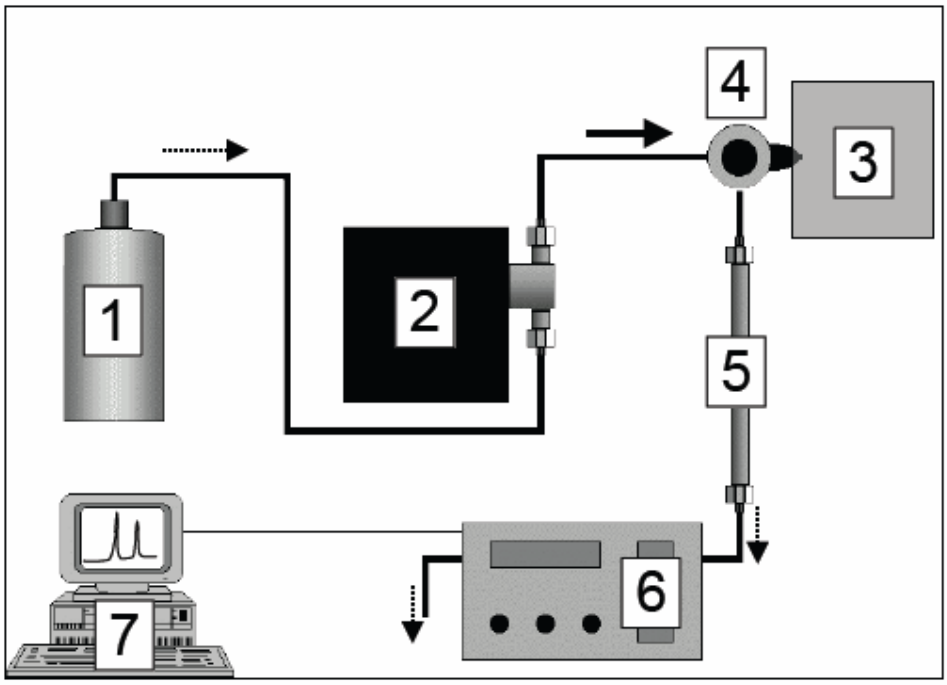


Figure 1: Schematic diagram of a basic HPLC system. The parts are (1) reservoir, (2) pump, (3) autosampler, (4) injection valve, (5) column, (6) detector and (7) data system.

1.1.2.3 Injector or Autosampler

Samples are usually introduced by syringe injection via a manual injector into the mobile phase stream or by the use of an autosampler. The important aspects in sample introduction are precise and reproducible injections. This is especially important with quantitative analysis where the reproducibility of the peak response is dependant on the precision of the sample introduction. Direct syringe injection through a manual injector was the first popular method of sample introduction. As HPLC instrumentation evolved, many autosampler techniques were applied so that sample introduction has become more precise and rapid [4].

1.1.2.4 Detector

HPLC detectors include ultraviolet-visible, fluorescence, electrochemical, refractometer, mass spectrometer and others. The UV visible absorption detector is the most widely used detector in liquid chromatography, since most organic compounds show some useful absorption in the UV region. This detector is fairly universal in application, although sensitivity depends on how strongly the sample absorbs light at a particular wavelength [5].

1.1.2.5 Solvent reservoir

Different containers are used as a solvent delivery system reservoir. The best material from which the containers are made is glass. Plastic containers are not recommended as it leads to plasticizer leaching. The container should be covered to prevent solvent evaporation. The tubing from the reservoir can be made of stainless steel or Teflon, and both are satisfactory [3].

1.1.2.6 Data handling and analysis

Data handling in HPLC is as important to the success of any experiment or analysis as any other components in the system. It is part of good HPLC techniques to properly label and document the analytical results. The advanced computer softwares used now in data handling and analysis allow easy recording and storage of all chromatographic data [6].

1.1.2.7 Calculation of peak parameters in HPLC

Chromatographic efficiency, expressed as the number of theoretical plates (N) was calculated based on the equation $N = 16\ (t_R/w)^2$, where N = number of theoretical plates, t_R = retention time, w = peak width at the base. Parameters are calculated as shown in Figure 2. Resolution was calculated using the equation $R_s = 2\ (t_{R2} - t_{R1}/w_2 + w_1)$. Asymmetry factor (AF) = A/B at 10% of peak height (A and B are the two half widths at each side of the peak centre) as shown in Figure 3.

1.1.3 Types of partition high performance liquid chromatography

1.1.3.1 Normal phase chromatography

Normal phase chromatography is characterized by the use of an inorganic adsorbent or chemically bonded stationary phase with polar functional groups and a non-aqueous mobile phase. This phase is consisting of one or more non-polar organic solvents.

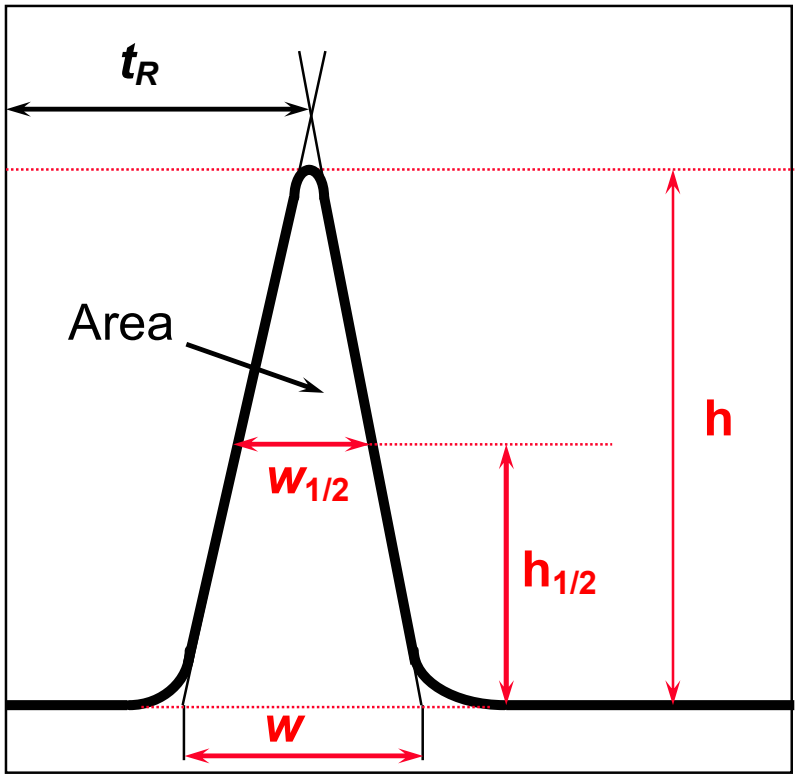


Figure 2: Representative diagram showing how peak parameters were calculated.

The retention of a solute is essentially determined by the balance of interactions it experiences in the mobile phase and its competition with mobile phase molecules for adsorption sites at the surface of the stationary phase [7].

1.1.3.2 Reversed phase liquid chromatography (RPLC)

The popularity of reversed phase liquid chromatography is easily explained by its unmatched simplicity, versatility and scope. Neutral and ionic analytes can be separated simultaneously. Retention in RPLC is believed to occur through non-specific hydrophobic interaction of the solute with the stationary phase. The near universal application of RPLC stems from the fact that almost all organic compounds have hydrophobic regions in their structure and are capable of interacting with the stationary phase.

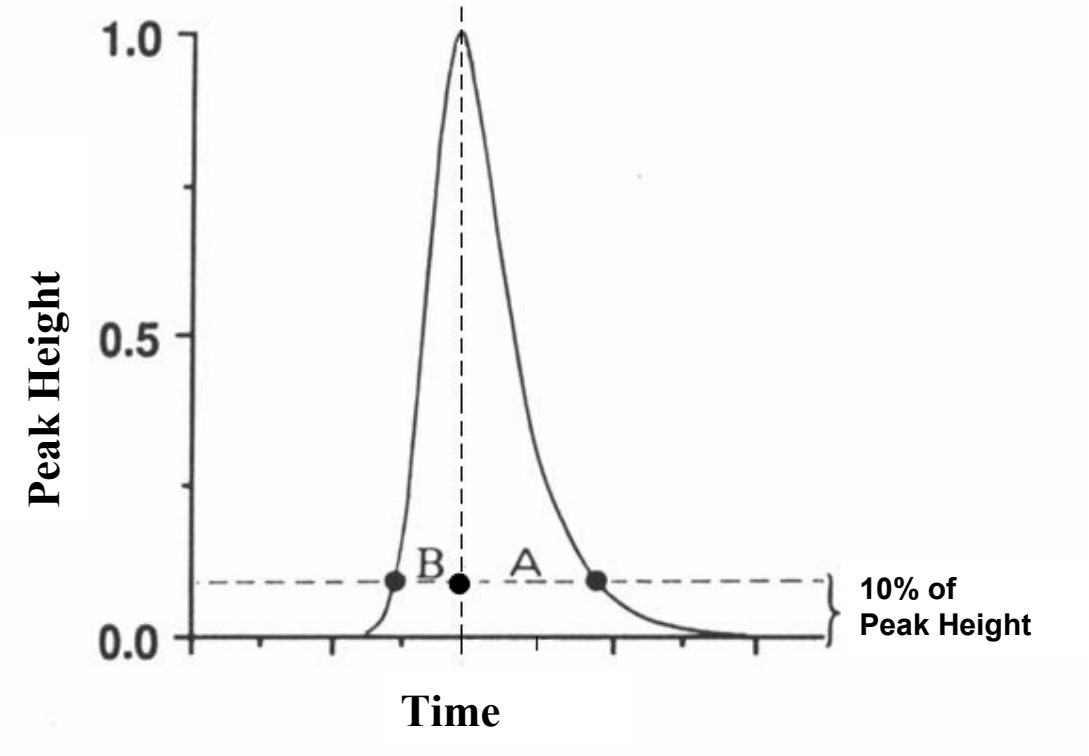


Figure 3: Representative diagram showing the method used for calculating peak asymmetry factor as recommended by USP. The asymmetry factor (AF) is equal to the ratio A/B.

A decrease in the polarity of the mobile phase leads to a decrease in retention. It is also generally observed in RPLC that branched chain compounds are retained to a lesser extent than their straight chain analogues and that unsaturated compounds are eluted before their fully saturated analogs [1].

A wide variety of RP-HPLC columns are available. Most columns are silica based. Silica offers good mechanical stability. A typical stationary phase is formed by chemically bonding a long-chain hydrocarbon group to porous silica. Typical ligands are n-octadecyl (C18), n-octayl (C8), n-butyl (C4), diphenyl, and cyano propyl [8].

1.1.3.2.1 Parameters affecting separation

Separation in reversed phase chromatography is affected by stationary phase type and column length. It is also affected by organic solvent type and percentage in the mobile phase and by mobile phase pH. Flow rate could also affect separation in reversed phase chromatography; however it is usually limited by the developed backpressure. Moreover temperature of the column also has an effect on separation. Parameters affecting separation in reversed phase chromatography are shown schematically in Figure 4.

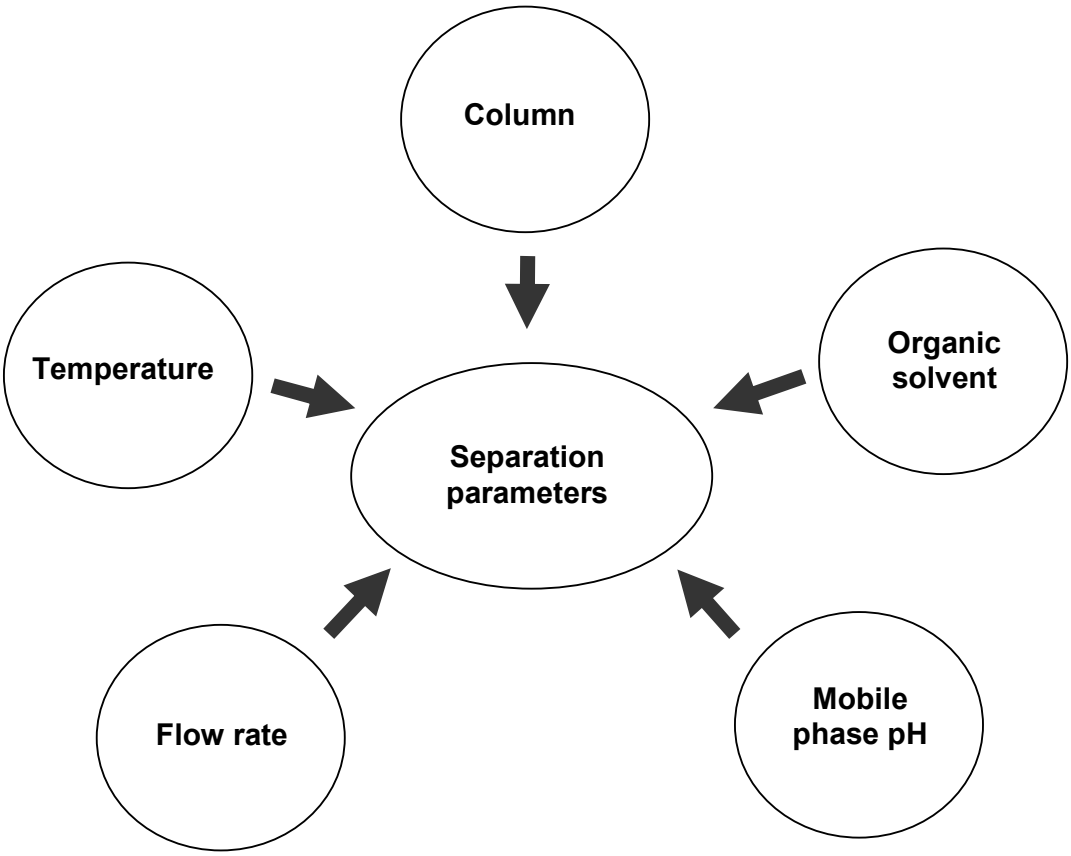


Figure 4: Parameters affecting separation in reversed phase chromatography.

1.1.3.2.2 Silica-based columns for RP-HPLC

The first introduced materials were based on irregular shaped silica particles, which had been bonded with a range of alkyl-bonded silanes having a carbon side chain. There are three common chain lengths, C4, C8, and C18. The octadecylsilyl ($C_{18}H_{37}$-, ODS) alkyl chain, rapidly became the most popular. The next development was the use of end capping, where a smaller reagent as trimethylsilyl chloride is used to cap the free silanol groups of the silica surface [9].

Nowadays, columns are packed with spherical silica particles. The beads or particles are generally characterized by particle size. Particle sizes generally range between 3 and 50 µm, with 3 to 5 µm particles being the most popular for analytical columns. Larger particles will generate less system pressure and smaller particles will generate more pressure. However, the smaller particles generally give higher separation efficiencies. Column dimensions are described by the column length, either in cm or mm, and the columns inside diameter in mm. The size of the column is determined by the particle size of the stationary phase. For example, 3 µm particles are packed in shorter column lengths because a 250 mm column would generate too much back pressure for a typical HPLC system. Also, because the 3 micron particles are very efficient, a shorter column probably has enough theoretical plates for the required separation. The typical analytical column inside diameter is 4.6 mm.

1.1.3.2.3 Isocratic vs. gradient elution

When the composition of the mobile phase is held constant, the practice is called isocratic elution. In contrast, in gradient elution, which is often preferred for separation of complex mixtures, the composition of the mobile phase is changed stepwise or linearly by mixing two or more solvents. The use of binary, ternary, and quaternary solvent gradients is fairly common [6]. The usual reason for choosing gradient elution is that the sample has a wide retention range were no isocratic condition result in $0.5 < k < 20$ for all bands of interest.

Samples of large molecules especially biological and those containing late interferences that can either foul the column or overlap with subsequent chromatograms also sometimes require gradient elution. An initial gradient elution run is often the best starting point for HPLC method development even when a final isocratic method is possible.

Many disadvantages have been reported for gradient elution. First, gradient equipment is not available in some laboratory. Furthermore, it cannot be used with some HPLC detectors e.g. refractive detector and do not always transfers well because difference in equipment can cause change in separation. Baseline problems are more common with gradient elution. In addition gradient elution takes longer runtime because of the need for column equilibrium after each run. Nowadays, there are many types of software for computer assisted method development. However, they still face many problems. For example, it needs an equipped HPLC system to perform these experiments automatically. Furthermore, failure of good column equilibrium creates a problem when a computer is used to predict retention and separation on the basis of prior runs. For these reasons and others the step by step approach is more favourable which allows interpreting the chromatograms from prior runs before choosing conditions for the next runs.

1.1.3.2.4 Classical method development strategy for RP-HPLC

The main steps for method development are shown in Chart 1. Before the development of a method the goals should be clearly defined. The goal could be assay of an active substance, so that the separation of other substances is not necessary. When the goal is the assay of impurities or other degradation products, a high detection sensitivity and resolution of all sample components should be planned. The goal could also be isolation or quantitation of the sample components. Furthermore, certain desires could be taken in account when developing a new method such as a short analysis time. The next step is the determination of sample information. In this context method development for regular neutral or ionic (acidic, basic, amphoteric or organic salt) samples will be discussed. Special samples as enantiomers which require chiral conditions will be out of this investigation. Also very hydrophilic samples may not have sufficient retention in RPLC even with mobile phase containing little or no organic solvent so that normal phase separation is required. One should try to collect as much as possible of sample information. This includes the number of compounds in the sample, the chemical structure of all or some of them, the molecular weight of the compounds, the pK_a values of the individual compounds, UV spectra, concentration range of the compounds and the sample solubility.

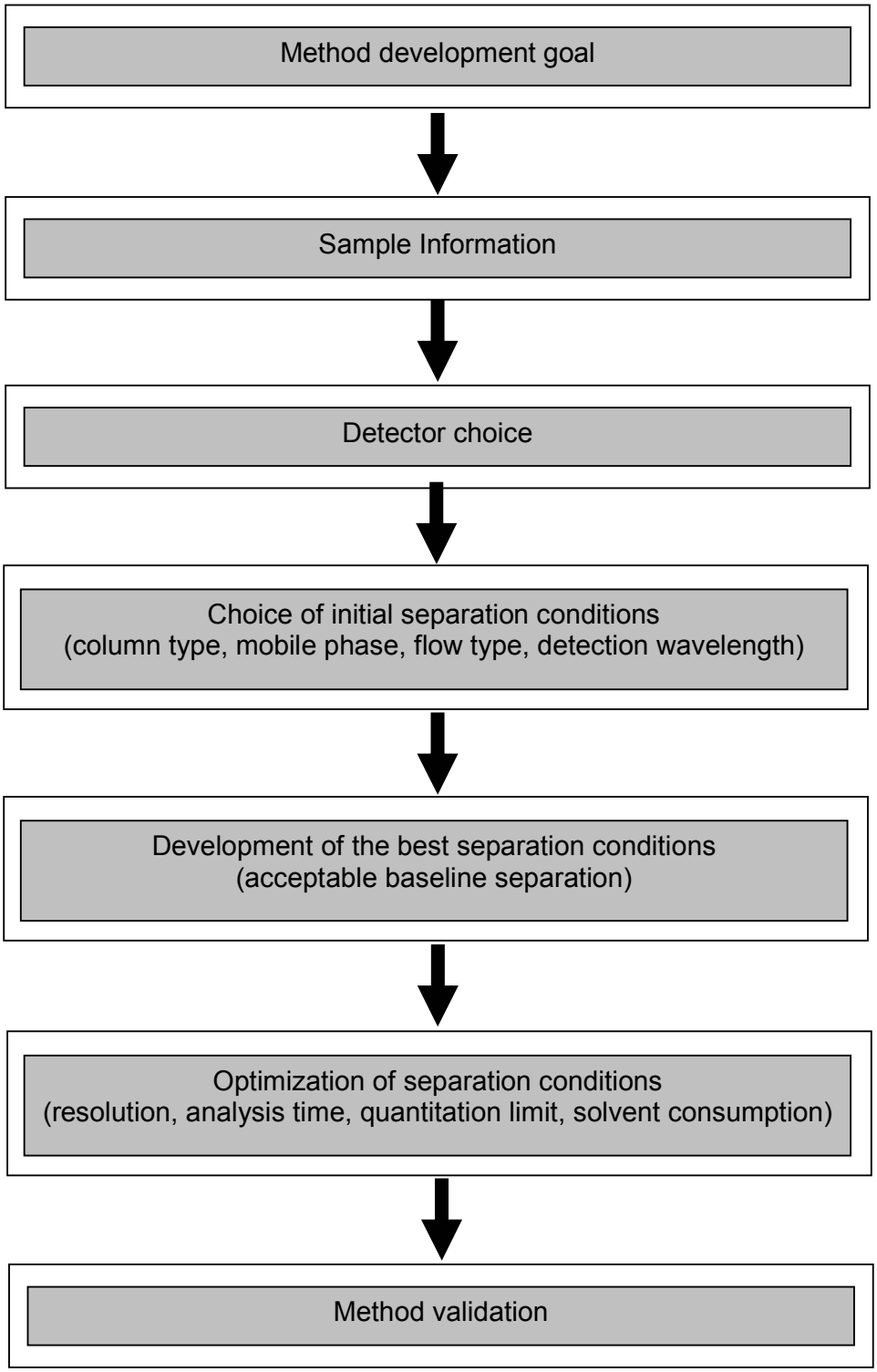


Chart 1: Main steps for method development in reversed phase HPLC.

Physicochemical properties of the sample components help to select proper buffer system and to guess about a suitable starting mobile phase based on hydrophobicity. UV spectra help to determine the maximum wavelength for detection. However, if no information is available (unknown sample) one could initially detect at 210 nm and afterwards adjust the detection wavelength depending on spectra provided by a diode-array detector (DAD). Accordingly it is useful but not essential to know the chemical structure and properties of the various sample compounds which helps in sample pre-treatment and detection. In principle, method development for any regular sample can be carried out in the same way regardless of whether the sample composition is known or standards are available. The composition of sample solvent should preferably be the mobile phase or a solution of lower elution strength to minimize baseline upset, decrease peak width and get better resolution.

Sample volume and mass are better to be small to avoid column overload. Even when a small volume is injected it is possible because of the mass of the sample to overload the column and so to broaden and change peak shape. This happens because the stationary phase can become saturated with the sample.

Especially sample volumes and masses of basic compounds should be small on silica columns to avoid band broadening and tailing as a result of silanol interactions. Increasing the sample size decreases N, t_R and R_s and gives broad peaks. However, the largest possible sample may be needed to increase detection sensitivity. One should be sure that the detector will detect all sample components of interest. The diode-array detector (DAD) is the first choice detector as it is applicable for most samples either directly or after derivatization. UV spectra can be found in literature or estimated from chemical structure or measured directly using UV spectrophotometer if pure compounds are available. However they can also be obtained during HPLC separation if DAD is available to give the UV scan for pure peaks. In such case, one could start the HPLC separation at 210 nm at which most of the compounds should have certain absorption. Another advantage of using a DAD detector instead of a simple UV-visible one is the ability to check peak purity and even peak identity if one has a standard UV spectra library. If UV response is inadequate one should use other detectors as fluorescence or electrochemical ones or derivatize the sample. The separation mechanism involves sample partition between a polar mobile phase

(water-organic) and a non-polar stationary phase. The results are different retentions of sample components according to the hydrophobicity of the sample. The less hydrophobic components elute first. An increase of solvent strength will decrease mobile phase polarity and so decrease the retention of compounds. The development strategy involves the start with a high percentage (not more than 95%, usually 80%) of organic modifier (e.g. acetonitrile or methanol) followed by successive runs with a 10 or 20% decrease of organic modifier in each run (down to not less than 5%) until all the peaks of interest are resolved within an acceptable run time and with the best possible resolution.

Generally one should not use more than 95% water in the mobile phase because the bonded phase can collapse if too much water is present, thus, it would change the retention characteristic of the column. On the other hand, higher percentage of organic modifier could affect the solubility of the buffer in the mobile phase. The change of solvent type (e.g. 40% ACN by 40% MeOH) or using a mixture of two organic solvents (e.g. 20% ACN + 20% MeOH) will probably have a greater effect on selectivity than changing solvent strength (solvent type selectivity). The use of amine modifier will improve the peak shape of basic samples when working at high pH due to blockage of ionized silanol by amines. The aqueous part of the mobile phase should be buffered at a certain pH especially for ionic samples. Changing the pH of the aqueous part of the mobile phase is another possible step to achieve good resolution. Most pH related changes occur within ± 1.5 unit of the pK_a value, outside this range the compound is almost completely ionized or non-ionized, so the retention does not change significantly with pH change. In RP retention increases when the compound becomes non-ionized. Most acidic drugs have pK_a 4 - 5 and basic pK_a 8 - 11. One should note that some acidic or basic compounds undergo a change in absorbance as pH is varied so that the band size changes. As the compounds become more ionized the retention time decreases e.g. for acidic compounds the retention time decreases at a high pH value of the mobile phase. Under non-ionizable condition the compound solubility in water deceases, so requiring the use of higher percentage of organic modifier in the mobile phase. An exception is given by sample matrices tending to precipitate at this pH (at which the compounds are non-ionized). In this case one should work under ionizable conditions to insure reproducible results not only for ionic samples, but also for neutral ones, because they may contain ionic

species. It is better to measure the pH of the aqueous part of the mobile phase before mixing with the organic solvent even though the solution ionization property may change after mixing with organic solvent. This is because electrode response for a mixture of aqueous and organic mobile phase tends to drift and so it is more difficult to get reproducible result. In silica based columns basic compounds can interact with ionisable silanols groups leading to increased retention, tailing and column to column irreproducibility. Protonated base (BH^+) in sample exchange with sodium, potassium, or other cations that are attached to ionized silica. All silica based columns contain accessible silanols, but their effect on sample retention can be reduced by one of the following ways: First, the use a low pH mobile phase ($2 < pH < 3.5$) to minimize the number of ionized silanols. Second, by working at a high pH at which basic compounds are not protonated (but this interferes with the fact that silica based columns are not completely stable under high pH values). Third, the use of high concentration phosphate buffer; high buffer concentration may be valuable as buffer cations will compete with sample cations to the ionized silica. Fourth, use low sample amount for basic compounds. When mixing aqueous and organic modifier parts of the mobile phase, one should keep in mind several facts. A methanol-water mobile phase gives higher solubility for buffers (e.g. phosphate buffer), than acetonitrile-water. Buffers with potassium salts are more soluble than buffers with sodium salts. Citrate buffer show a high UV absorbance so limiting the UV detection to wavelengths above 230 nm. Phosphate buffers control pH in the range of 2.1 - 3.1 and 6.2 - 8.2 while acetate buffers work in the range 3.8 – 5.8. Care should be taken to avoid precipitation of the buffer at high percentage of organic modifier. This may require low buffer concentration (5 or 10 m*M*) or more soluble buffers. The separation achieved in the first runs usually will be less than adequate. Improving the separation in term of resolution or analysis time should be done to obtain a good developed method. In addition, further optimization of the resolution, analysis time and quantitation limit may be required. The final optimized method should then be validated according to the ICH requirements and valid pharmacopoeias [10].

1.1.4 Fast HPLC analysis

1.1.4.1 Definition

There is no clear definition for fast analysis. The term fast HPLC is a relative one. Fast analysis refers to decreasing the analysis time of a method. Analysis time by itself is sometimes a poor measurement of chromatographic parameters; rather the important parameter is the number of compounds separated per unit time. For example a 10 compounds running in ten minutes is more time efficient than a 2 compounds run in 10 minutes. Nevertheless, it should be noted that the terms “fast LC”, “high speed HPLC”, “fast HPLC” and “ultra-fast HPLC” are common place in the literature without a formal definition.

1.1.4.2 Factors affecting fast HPLC analysis

Factors affecting HPLC analysis are best described in Table 1. Several approaches have commonly been used for fast chromatography during the last decade. In general short column, high temperature, high flow rates all should lead to a faster HPLC analysis. Each of these parameters is interrelated with the dependant parameters of analysis time, column backpressure and column efficiency. Table 1 lists the relationship among these parameters [11]. However, the effect of temperature on analysis time and efficiency is sometimes variable and unpredictable.

Table 1: Relationship between the independent parameters: column length, flow rate, particle size and column temperature to the dependant parameters of analysis time, column backpressure and column efficiency.*

	Column Length (L)	Flow Rate (F)	Particle Diameter (d_p)	Column Temperature (T)
Analysis time	α L	α 1/ F	indirectly related	α $1/T^x$
Backpressure	α L	α F	α $1/(d_p)^2$	α 1/T
Efficiency	α L	by Van Deemter	α $1/d_p$	α T

*α means directly proportional

1.1.4.2.1 Short columns

The use of a short column reduces separation time. However, resolution will be reduced due to reduction of plate numbers at the same time. Half the column length means half the run time and half the number of plates. In general, column length is directly proportional to analyte retention time, column efficiency and backpressure. Reduction of column length is acceptable as long as column efficiency remains sufficient for separation. The use of small particles with short columns will compensate for reduced efficiency but will be limited again by the backpressure [12].

1.1.4.2.2 Increasing the flow rate

Increasing the flow rate is another way for reducing run time. Flow rate is inversely proportional to analysis time, so doubling the flow rate will result in halving the analysis time. An optimum flow rate has been established for common column diameters. These optimum flow rates correspond to (more or less) the highest column efficiency according to the Van Deemter plot. Doubling the flow rate saves as much time as using a column with half the plate length. Keeping in mind that, increasing flow rate also means less reduction of plate number than reducing column length [12].

Unfortunately, flow rate is also proportional to the pressure drop across the column, measured as system pressure or column backpressure. Most fast analyses operate above the optimum flow rate, typically at the highest flow rate possible within allowed column and system pressures.

1.1.4.2.3 Small particles

Columns with small particle sizes (e.g. 2 µm) will lead to a fast analysis due to the superior efficiency. However, this is very challenging as this approach result in high pressure and the columns are more likely to be blocked [12].

1.1.4.2.4 High temperature

High temperature is beneficial in at least three aspects. First, an increase in column temperature reduces the viscosity of the mobile phase and therefore the column backpressure, permitting faster flow rates. Second, an increase in column temperature deceases band spacing and so increases resolution between adjacent peaks. Third, an increase in column temperature enhances analyte mass transfer, increasing efficiency at faster flow rates. The use of increased column temperature is limited by the thermal stability of the analyte, the thermal stability of the stationary

phase and the boiling point of the mobile phase. Even with increasing the temperature, column backpressure remains the limiting factor. Therefore the traditional approaches to fast HPLC analysis are inherently restricted by column backpressure.

1.1.4.3 New trends for fast LC analysis

Nowadays, there is a competition between two means for fast LC analysis, namely, HPLC with monolithic phases and small particle phases used in ultra performance liquid chromatography (UPLC™). With both phase types a substantially higher column efficiency, analysis speed and sensitivity can be achieved. With UPLC™ this is achieved particularly by the use of small particles in the stationary phases. However, the use of small sub-2µm particles requires the use of ultra high pressure (approximately 1400 bar) demanding special high quality equipment to cope with the high pressures. While for UPLC™ many phases are already commercially available, the selection of available monolithic phases is still limited at present to standard materials such as RP-18 and RP-8. However, these phases do have a substantially higher permeability to build a much lower backpressure in comparison to the small particle phases used in UPLC. Therefore monolithic phases can be used with standard HPLC instruments.

1.1.4.3.1 Monolithic silica columns

1.1.4.3.1.1 Overview about monolithic silica columns

Monolithic silica columns were first introduced in 1991 by Minakuchi and Soga [13]. The preparation of these silica rod materials involved a sol-gel process using highly pure silica. The formed silica rod is then encased in poly ether ethyl ketone (PEEK) shrink-warp tubing, which prevents void formation. The obtained highly porous skeleton is characterized by a bimodal pore structure consisting of large macropores (diameter 2 µm), and mesopores (13 nm in diameter). The large macropores are responsible for a low flow resistance and therefore allow for the application of high eluent flow rates, while the small pores ensure sufficient surface area (300 m^2/g approximately) for separation efficiency. Monolithic columns also have a significantly higher total porosity after octadecylsilylation than conventional particulate columns, over 80% vs. ca 65%, respectively [14]. However, due to the fact that the density of monolithic columns is much lower, the loadability of a conventional column of the same size is much higher. The main parameters for a monolithic silica HPLC column

are summarized in Table 2 [15]. Till now the stationary phase availability of monolithic columns is limited to normal silica, C8 and C18. Furthermore, because of the significant shrinkage during the formation of the skeleton, it is difficult to prepare straight rods longer than about 15 cm, which limits the length of the final columns. However, it is possible to enhance the separation efficiency by coupling several monolithic columns together. Nowadays about 450 papers were published describing the use of monolithic columns in various fields. This includes drug analysis [16-19], food and environmental analysis [20, 21] and bio-analysis [22-24]. There have been many investigations into the feasibility and parameters during method transfer from traditional LC columns to monolithic columns [25-29].

Table 2: Specification for a Chromolith Performance HPLC column.

Silica type	High purity (99.99%)
Particle size	Monolithic
Macropore size	2 µm
Mesopore size	13 nm
Surface area	300 m^2/g
Total porosity	> 80%
Surface modification	RP-18 endcapped
pH range	2.0 - 7.5
Column cladding material	PEEK [poly(ether ethyl ketone)]

However, the number of developed methods using monolithic columns is much smaller than that using particle packed columns. Till now they are not mentioned in official methods of any pharmacopoeia. Furthermore, the transferability of analytical methods from conventional to monolithic columns and the precision of such columns are still under discussion.

1.1.4.3.1.2 Particle packed vs. monolithic silica columns

The use of conventional particle packed HPLC columns containing the classic 3 or 5 µm small silica particles often results in high back pressure. This high back pressure

may damage both the column and the HPLC system; therefore, classic HPLC columns have limited length and a limited number of theoretical plates. Increasing the plate count by using 2 or sub 2 µm particle size have been tried, but this results in unacceptable backpressure when normal HPLC instruments are used. Scientists wish to speed up the entire separation process and accelerate the analysis without much affecting the resolution or facing high system backpressure. High throughput analysis has become one of the most important issues in the high performance liquid chromatography. Laboratory automation of HPLC systems has come a long way toward improving sample throughput by enabling 24 hours a day operation. The systems however are still limited by the separation technology itself, that is, the separation column available. Monolithic silica columns are claimed to provide excellent separations in a fraction of time that a standard particulate (particle-packed) column will take, because they are made from high porous monolithic rods (Figure 5) with a bimodal pore structure.

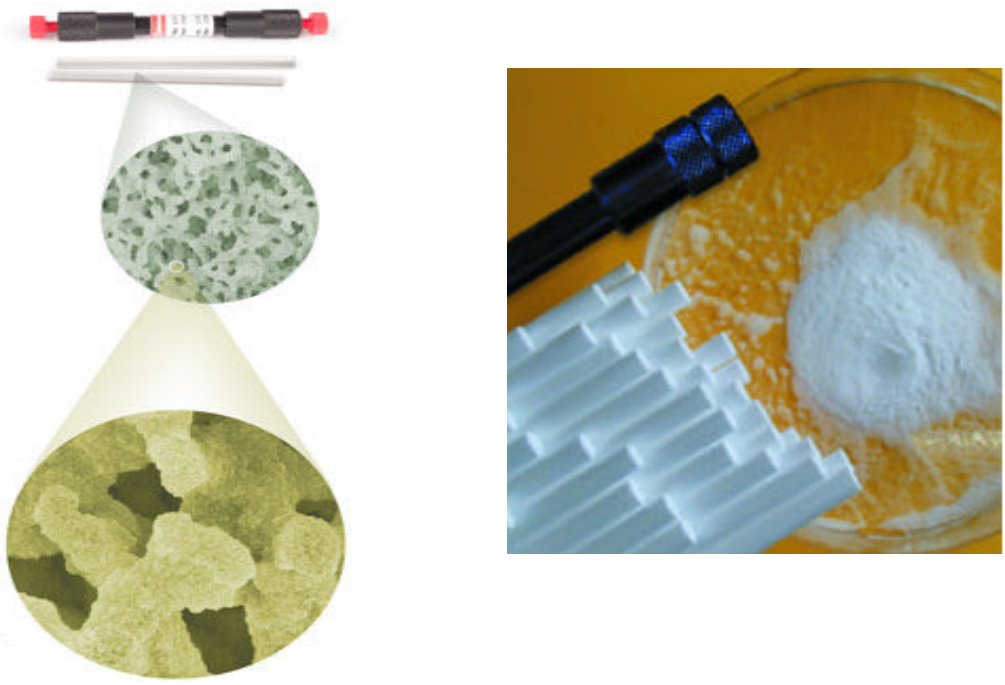


Figure 5: Monolithic porous silica bodies shaped as rod [30].

The column is no longer packed with small particles but consists of a single piece of high purity polymeric silica gel. High flow rates could be used with monolithic columns due to the high porosity of the column provided mainly with macropores. Besides, high efficiency is ensured by the mesopores that provide very large surface area for separation (Figure 6).

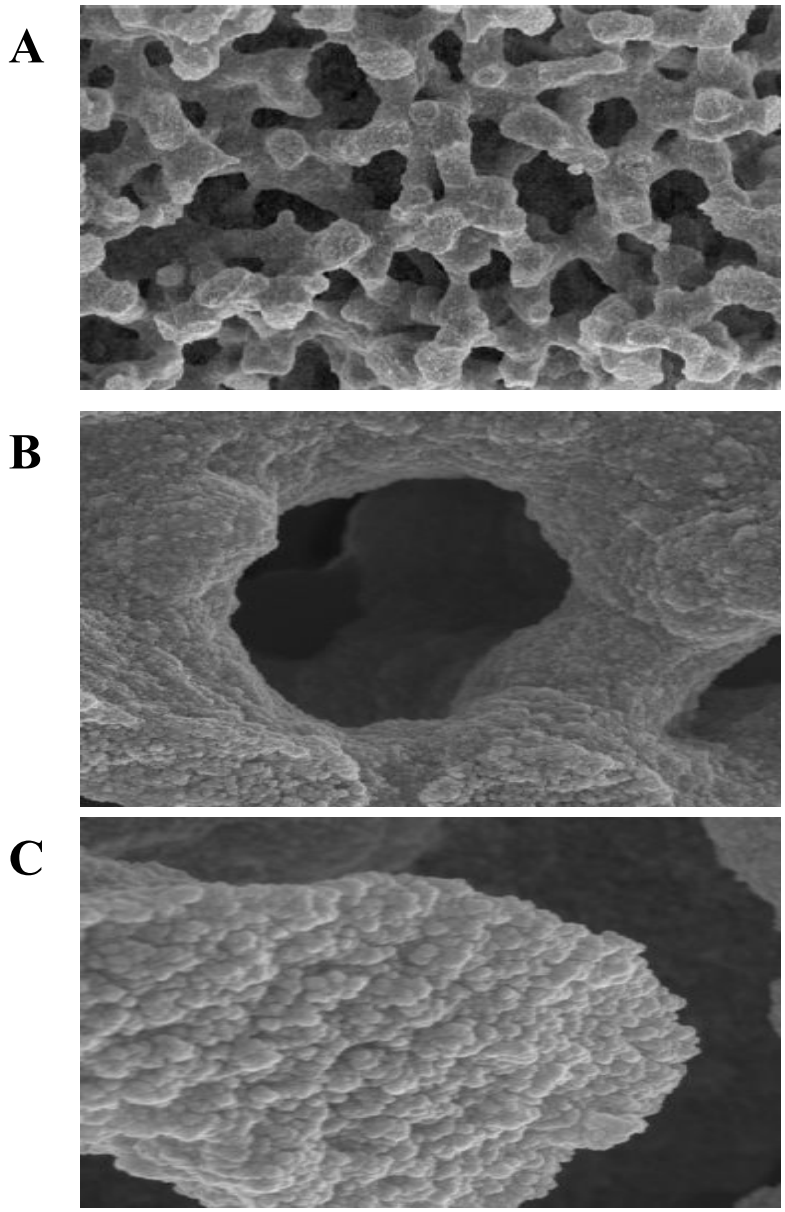
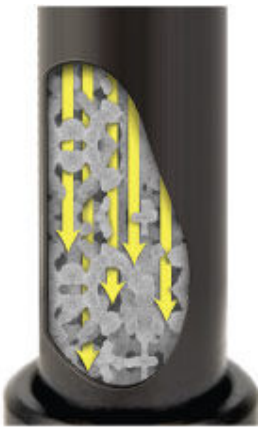


Figure 6: Monolithic Silica A, Macropores B, and Mesopores C [15].

The difference between monolithic and conventional particle-packed columns is shown in Figure 7.

Conventional Silica "Particle-Based"
➢ High flow resistance:
Limits ability to shorten run times
➢ High backpressure:
Reduces life of pumps, seals, and column



Monolithic porous silica rod
➢ High flow rates:
Significantly shorter run times
➢ Low backpressures:
Less stress on system and column

Figure 7: Representative conventional particle-packed vs. monolithic silica HPLC columns [31].

Furthermore, the separation efficiency of monolithic columns does not decrease significantly when the flow rate is increased, as is the case with particulate columns. Accordingly, it is possible to operate monolithic columns at high flow rates with minimal loss of peak resolution. It is also possible to connect monolithic columns together using a special column coupler to produce a column with high plate count at low backpressure for complex separation (Figure 8). High resistance to blockage and long column lifetime are also advantages of high porosity. Despite these promising characters more time is still needed to improve its applicability to official use and to evaluate result repeatability and batch to batch reproducibility and method transfer for different pharmaceutical compounds, in addition to method development using them [32].

Figure 8: Coupling of two Chromolith Performance RP-18e HPLC columns using the special monolithic column coupler [15].

1.1.4.3.1.3 Commercially available monolithic HPLC columns

The different types and dimensions of monolithic silica HPLC columns provided by Merck are summarized in Table 3 [15]. The trade name “Chromolith” indicates a monolithic material. The same types and dimensions are also available by Phenomenex Company under the trade name “Onyx” [31].

Table 3: Monolithic silica columns produced by Merck Company.

Column Description	Length	Internal diameter
Capillary columns		
Chromolith CapRodTM 150 - 0.1 mm RP-18 endcapped	150 mm	0.1 mm
Analytical columns		
Chromolith Performance 100 - 4.6 mm RP-18 endcapped	100 mm	4.6 mm
Chromolith SpeedROD 50 - 4.6 mm RP-18 endcapped	50 mm	4.6 mm
Chromolith Flash 25-4.6 mm RP-18 endcapped	25 mm	4.6 mm
Chromolith Performance 100 - 4.6 mm RP-8 endcapped	100 mm	4.6 mm
Chromolith Performance 100 - 4.6 mm Si	100 mm	4.6 mm
Chromolith Column Coupler		
Chromolith Validation Kit RP.18E (3 columns from different batches)	100 mm	4.6 mm
Guard columns		
Chromolith RP-18 endcapped Guard column 5 - 4.6 mm	5 mm	4.6 mm
Chromolith RP-18 endcapped Guard column 10 - 4.6 mm	10 mm	4.6mm
Semi-preparative and preparative columns		
Chromolith SemiPrep 100 - 10 mm RP-18 endcapped	100 mm	10 mm
Chromolith prep 100 - 25 mm Si	100 mm	25 mm
Chromolith prep 100 - 25 mm RP-18 endcapped	100 mm	25 mm

1.2 Capillary electrophoresis

Capillary electrophoresis is a family of related separation techniques that use narrow-bore fused silica capillaries to separate a complex array of large and small molecules. The variations include separation based on size and charge differences between analytes (termed Capillary Zone Electrophoresis, CZE, or Free Solution Capillary Electrophoresis, FSCE), separation of neutral compounds using surfactant micelles (Micellar Electrokinetic Chromatography, MEKC) sieving of solutes through a gel network (Capillary Gel Electrophoresis, CGE), and separation of zwitterionic solutes within a pH gradient (Capillary Isoelectric Focusing, CIEF). CZE and MEKC are the most frequently used separation techniques in pharmaceutical analysis. CGE and CIEF are of importance for the separation of biomolecules such as DNA and proteins, respectively and are of increasingly importance as development of biotechnology derived drugs becoming more frequent [33].

1.2.1 CE instrument

The basic instrumental configuration for CE is relatively simple. All that is required is a fused-silica capillary with a narrow bore (typically 25 - 100µm) and with an optical detection window, a controllable high voltage power supply, a two electrodes assembly, two buffer reservoirs, and a detector e.g. an ultraviolet (UV) one. The ends of the capillary are placed in the buffer reservoirs and the optical detection window is aligned with the detector. After filling the capillary with buffer, the sample is injected by pressure or electrokinetically. Finally the selected voltage is applied to run the buffer and analytes. A simplified diagram of a CE instrument is illustrated in Figure 9.

1.2.2 Capillary zone electrophoresis

Capillary zone electrophoresis (CZE), also known as free solution capillary electrophoresis, is the simplest form of CE. The separation mechanism is based on differences in the charge-to-mass ratio. The homogenity of the buffer solution and the constant field strength throughout the length of the capillary are important aspects in CZE. Capillaries are typically in the range of 25 - 100 µm inner diameter and 0.2 to 1.0 m in length. The applied potential is typically 10 to 30 kV. Due to the electro-osmotic flow sample components migrate towards the negative electrode. A small volume of sample (typically about 10 nl) is injected at the positive end (anode) of the capillary and the separated components are detected near the negative end (cathode) of the capillary. CE detection includes absorbance, fluorescence,

electrochemical, and mass spectrometer detectors [34]. Following injection and application of voltage, the components of a sample mixture separated into discrete zones as shown in Figure 10.

1.2.2.1 Electro-osmotic flow

The surface of the silicate glass capillary contains negatively-charged silanol functional groups (at pH > 2) that attract positively-charged counter ions. The positively-charged ions migrate towards the negative electrode and carry solvent molecules in the same direction.

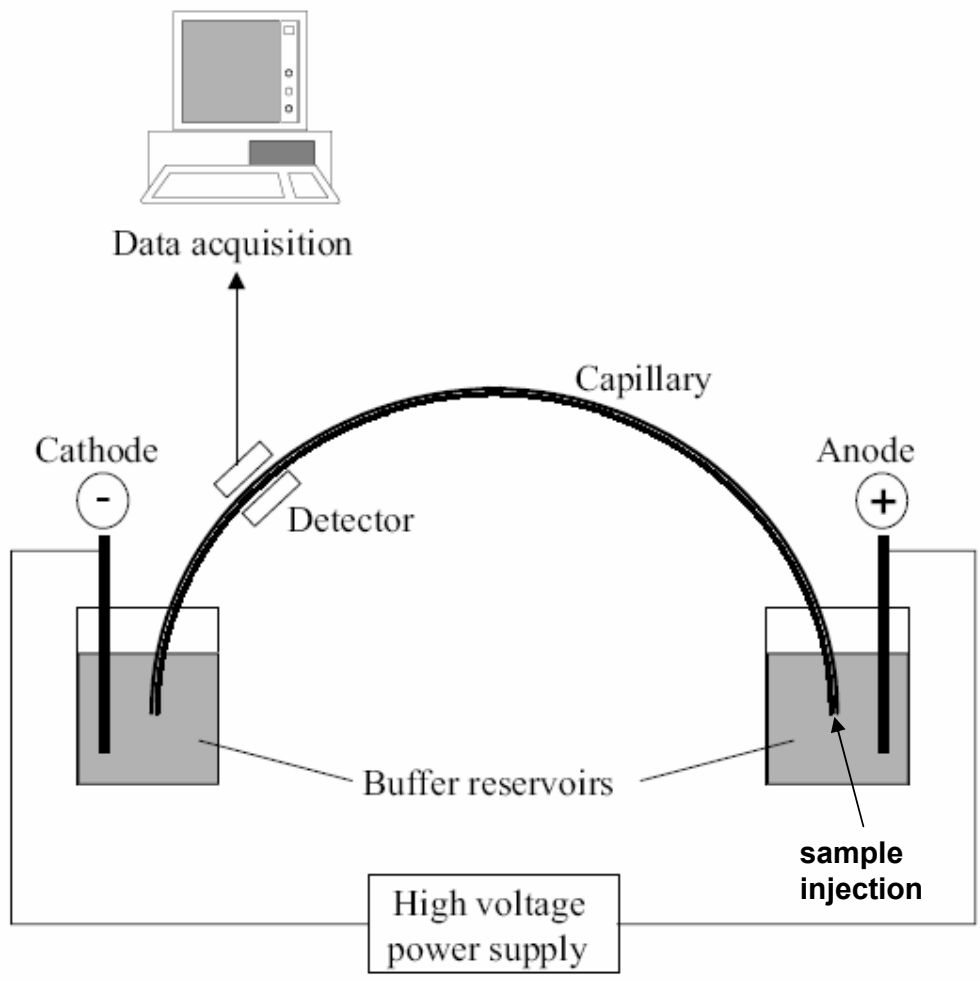


Figure 9: Schematic diagram of a basic CE system.

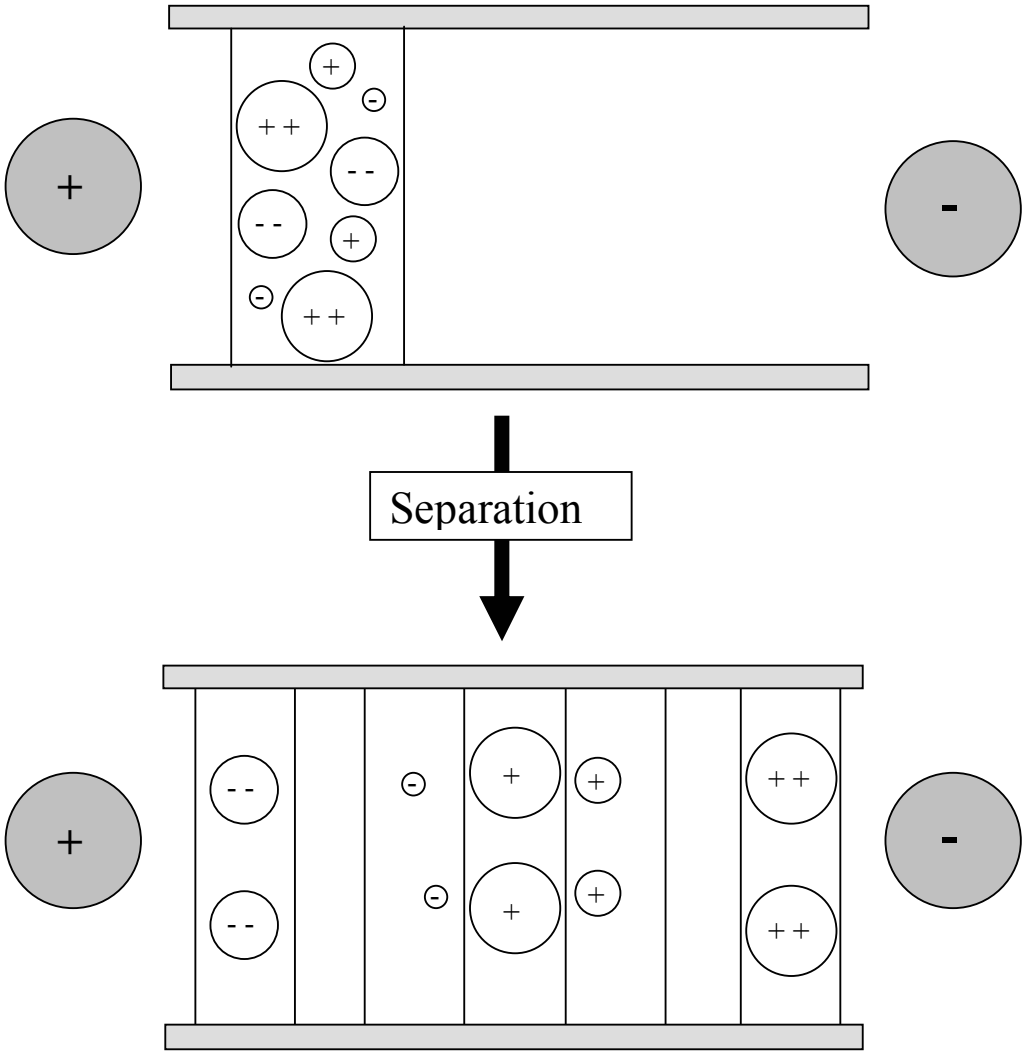


Figure 10: Separation of charged species by capillary zone electrophoresis.

This overall solvent movement is called electro-osmotic flow (Figure 11). During a separation, uncharged molecules move at the same velocity as the electro-osmotic flow. Positively-charged ions move faster and negatively-charged ions move slower. At high pH, the EOF is large and at low pH it is very small. This is related to the degree of silanol group ionization on the silica of the capillary surface (Figure 12). When applying a pressure driven system such as in liquid chromatography, the frictional forces at the liquid solid interface result in a laminar or parabolic flow profiles. As a consequence of parabolic flow, a cross sectional flow gradient occurs in the tube resulting in a flow velocity that is highest in the middle of the tube and approaches zero at the tubing wall.

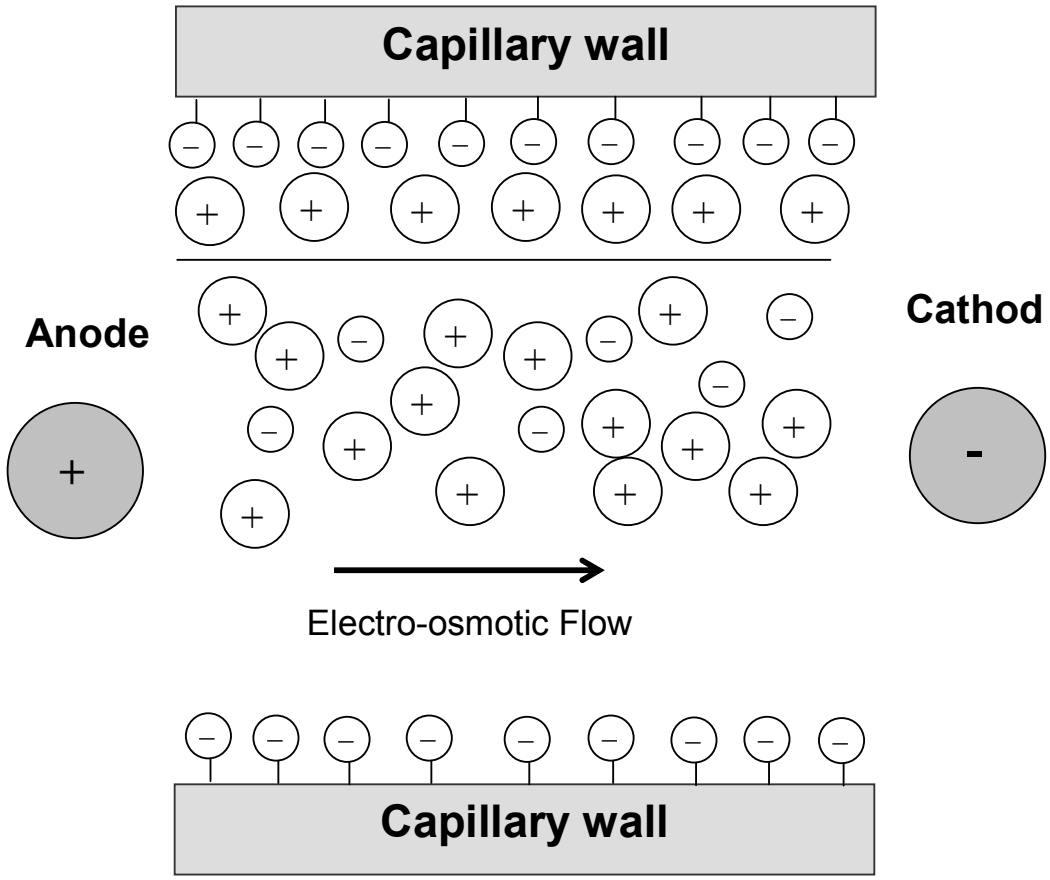


Figure 11: Electro-osmotic flow inside capillary.

This velocity gradient results in substantial band broadening. In electrically driven systems as CE, the driving force of the EOF is uniformly distributed along the entire diameter of the capillary. As a result the flow velocity is uniform across the entire tubing diameter except very close to the wall where the velocity again approaches zero (Figure 13) [34].

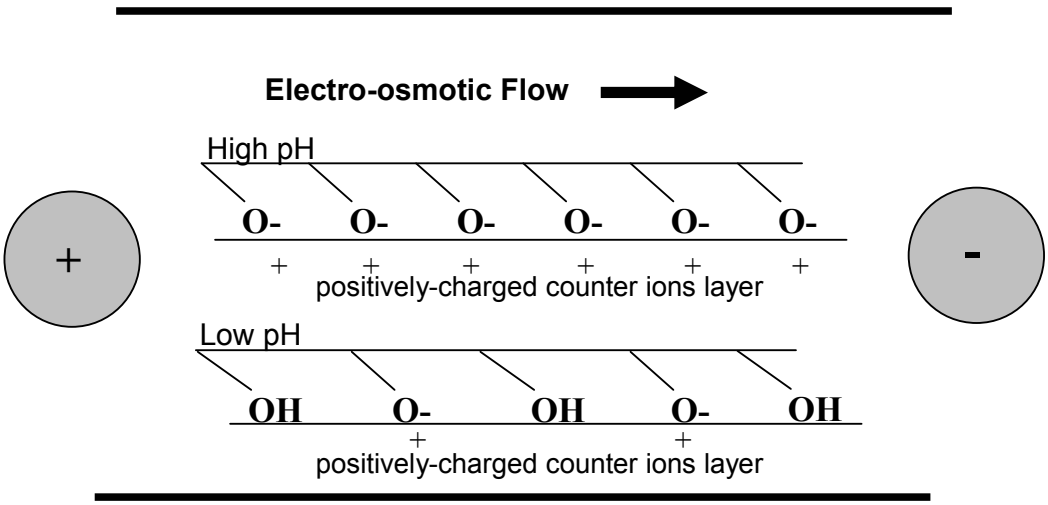


Figure 12: Effect of pH on the electro-osmotic flow inside the capillary.

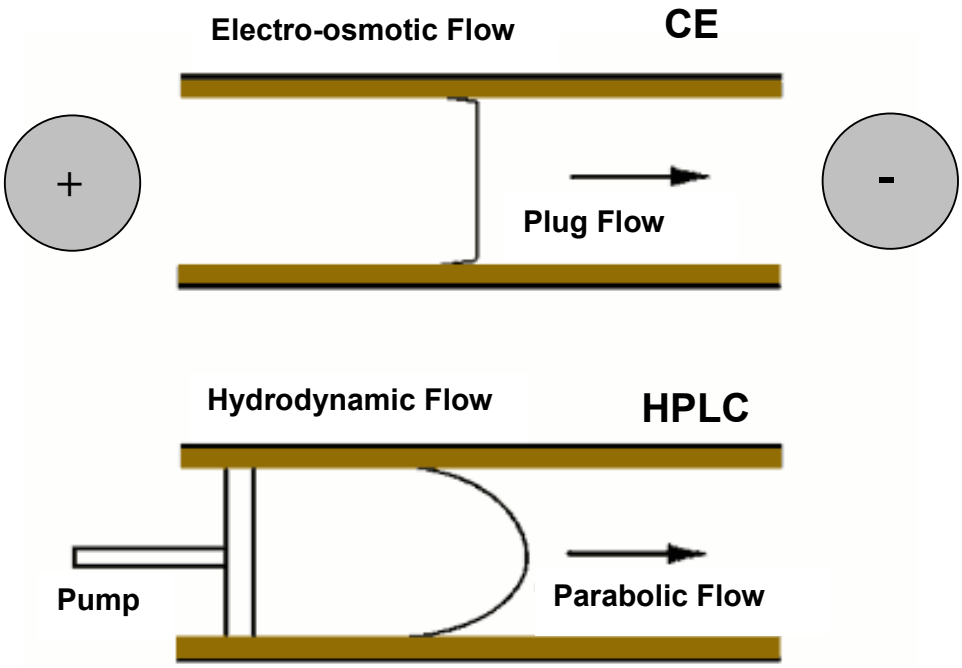


Figure 13: Flows in CE and HPLC.

1.2.2.2 Sample stacking

One of the simplest methods for sample pre-concentration is to induce stacking of the sample components this is easily accomplished by exploiting the ionic strength differences between the sample matrix and separation buffer. When the sample matrix is of lower ionic strength than the separation buffer (ten folds has been recommended), the sample components stack at the interface between the sample plug and the separation buffer (Figure 14). This results from the fact, that sample ions have an enhanced electrophoretic mobility in a lower conductivity environment. When a voltage is applied to the system, sample ions in the sample plug instantaneously accelerate towards the adjacent separation buffer zone where, on crossing the boundary, the higher conductivity environment induces a decrease in electrophoretic velocity and subsequent stacking of the sample components into a smaller buffer zone than the original sample plug. Within a short time, the ionic strength gradient dissipates and the charged analyte molecules begin to move from the stacking sample zone towards cathode.

1.2.2.3 Electrode polarity

Establishing the electrode polarity is important in capillary zone electrophoresis and is the default initial setting to be noted before the beginning of analysis. The normal polarity for CE is to have the anode (+) at the inlet and cathode (-) at the outlet. In this format, EOF is towards the cathode (detector/outlet). This is the standard polarity for most modes of CE. If set in the reversed polarity (cathode at inlet; anode at outlet), the direction of EOF is away from detector and only negatively charged analytes with electrophoretic mobility greater than the EOF will pass the detector [35].

1.2.2.4 Applied voltage

Usually a voltage between 10 to 30 kV is used. Increasing the voltage will increase the sample migration and electro-osmotic flow, as well as shorten analysis time. It may also increase the sharpness of the peaks and improve resolution [35]. However, the advantages associated with increasing the voltage may be lost if the sample matrix ionic strength is much greater than the running buffer ionic strength and/or the increased production of Joule heat cannot be efficiently dissipated.

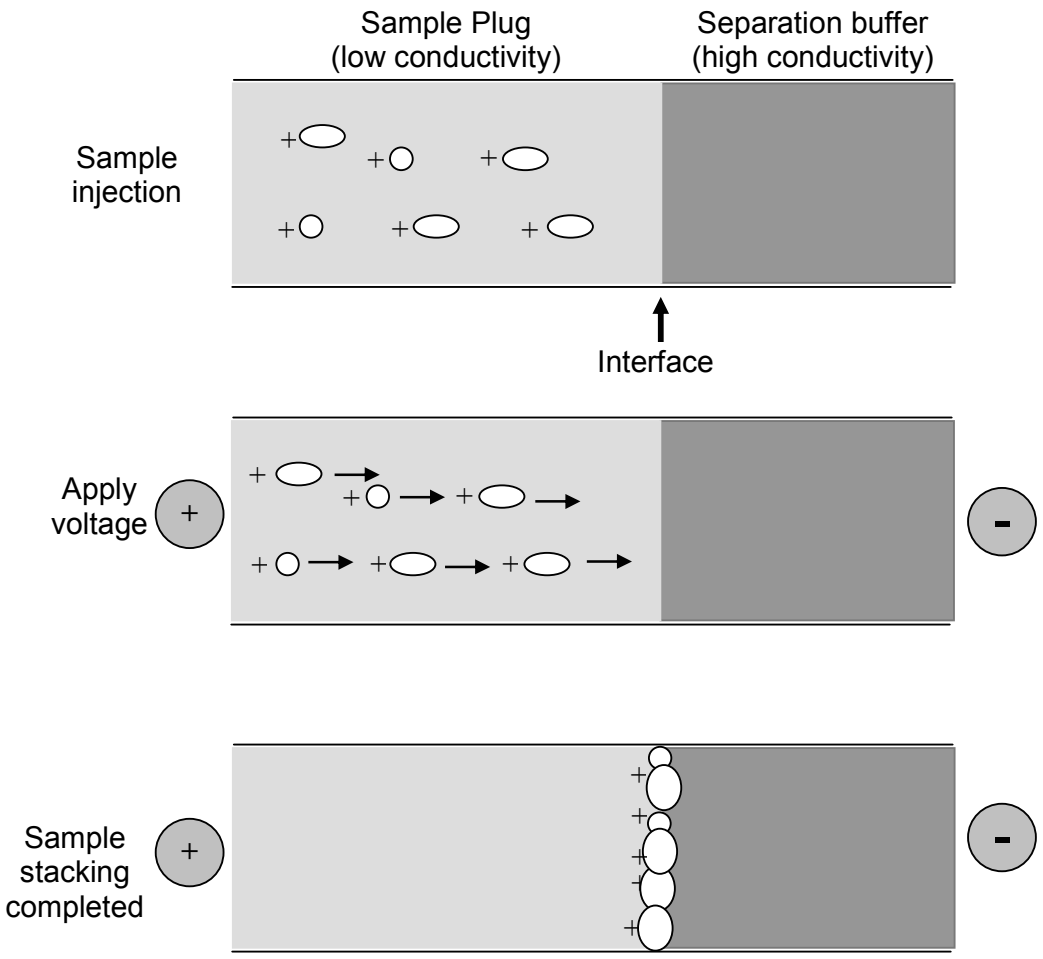


Figure 14: The sample stacking mechanism.

1.2.2.5 Temperature effect

Joule heating of the capillary results in a decrease of solution viscosity. This leads to a further increase in EOF, ion mobility, and analyte diffusion, which may ultimately result in band broadening. Joule heating is a consequence of the resistance of buffer to the current. Separation should be done with thermostatted capillary at close to ambient temperature. Whenever temperature control starts to be a problem, the usual strategy is to use a smaller bore capillary (less current reduces the heat production) or longer capillary (more surface area dissipates the heat generated).

1.2.2.6 Effect of capillary dimensions on separation

1.2.2.6.1 Internal diameter

Increasing the capillary internal diameter improves detection sensitivity (path length will be longer). However, this leads to less efficient dissipation of heat which then results in a temperature gradient across the capillary and band broadening due to thermal effects.

1.2.2.6.2 Length

Increasing the capillary length will increase migration (analysis) time. The increased length has no significant effect on separation in most cases. Exceptions are in chiral separation using CDs and in MEKC. In these cases the CDs and/or the micelles act as a pseudo-stationary phases and thus longer capillary allows more interaction. Also, as the length increases, there will be a concomitant decrease in the electrical field strength at constant voltage and, hence, higher voltage could be applied.

1.2.2.7 Effect of pH

Increasing the separation buffer pH will result in an increase in EOF. The buffer pH may be altered by other parameters such as temperature, ion depletion of the buffer (caused by repetitive use of the same separation buffer) and organic additives. A wide variety of buffers can be employed in CZE. Phosphate buffers are used around pH 2.5 and pH 7.0, and borate around pH 9 with a typical buffer concentration is 50 - 100 m*M.*

1.2.2.8 Ionic strength

The choice of the buffer species can have a dramatic effect on separation by CE. Furthermore, increasing the ionic strength of the separation buffer has the effect of decreasing EOF and hence, increasing the separation time. Increasing the ionic strength will also increase the current at a constant voltage to the point where adequate thermostatting of the capillary becomes a concern. The advantage of having a high ionic strength, in addition to the obvious improvement in buffer capacity, is the reduction of analyte-wall interactions [36]. The net effect on the separation will be to improve resolution. It is provided that capillary thermostating capacity is not exceeded and the unwanted analyte degradation processes do not occur.

1.2.2.9 Buffer additives

The addition of organic modifier to the separation buffer will have different effects, depending on the nature of the additive. One of the effects that often results is a change in EOF. For example, the addition of 1,4-diaminobutanes and diamino-alkanes to the buffer has been proposed to enhance resolution by slowing EOF through a dynamic (non covalent) coating of the capillary wall, thus improving the resolution of proteins [37]. Other additives have the effect of decreasing both the conductivity of the buffer and EOF. In such cases the subsequent enhancement in resolution may be a combination of the slower EOF (i.e. increased migration time), decreased thermal diffusion, and improved analyte solubility. Methanol in buffer can increase the solubility and resolution of the analytes [38].

1.2.3 Enantiomeric separation using capillary zone electrophoresis

1.2.3.1 Introduction to enantiomeric drugs

Many drugs in use (about 40%) are known to be chiral. It is well established that the pharmacological activity is often restricted to one of the enantiomers. In several cases unwanted side effects or even toxic effects can occur with the second enantiomer. Even if the side effects are not that drastic, the inactive enantiomer has to be metabolized and represents an unnecessary burden for the organism. The administration of the pure pharmacologically active enantiomer is therefore of great importance. The development of enantiomer separation methods for controlling synthesis, for enantiomeric purity check, and for pharmacodynamic studies is attracting increasing interest. Recently, pharmaceutical companies try to carry out stero-selective reactions in order to produce the pure wanted enantiomer. However these stero-selective reactions do produce some enantiomeric impurities. The importance of stereochemistry, and also the need for enantioselective analysis methods, has become well recognized [39]. Enantiomers are stereoisomers that display chirality that is having one or more asymmetric carbon centers, allowing them to exist as non-super-imposable mirror images of one another. These isomers are difficult to analyze as they are both physically and chemically identical in an achiral environment and differ only in the way they bend plane-polarized light and in their behaviour in a chiral environment. The first step toward separating enantiomers is to create diastereomers. Diastereomers may be created through chemical derivatization

with a "chiral" reagent, or they may be formed transiently through interactions with chiral selectors. The latter is usually the most desirable as it is the easiest to employ.

1.2.3.2 Methods for separation of enantiomeric drugs

Different chromatographic separation techniques have been reported for chiral separation. Those include high-performance liquid chromatography (HPLC) both reversed phase [40-46], and normal phase chromatography [47-51], supercritical-fluid chromatography (SFC) [52, 53], and capillary electrophoresis (CE) [54-63]. In HPLC a special chiral bounded stationary phase column mainly of cyclodextrin is used. SFC uses an eluent of carbon dioxide and a high viscous chiral based liquid stationary phase. During recent years, it has been shown that capillary electrophoresis (CE) is an excellent technique for chiral separation.

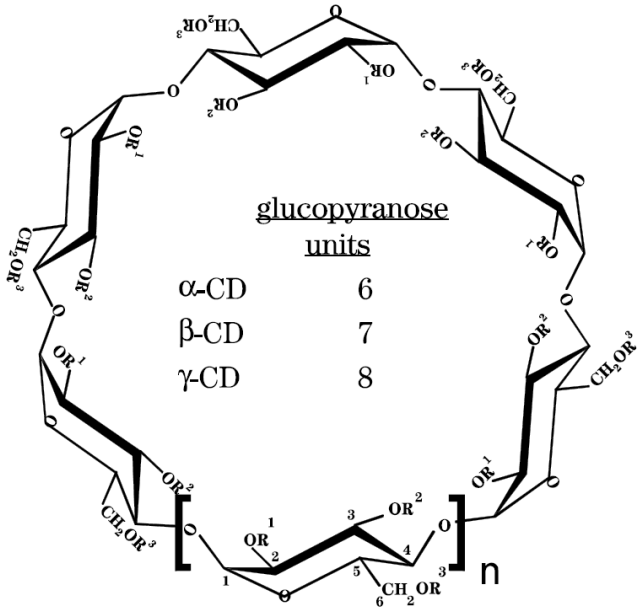
The main advantages of this technique are the high efficiency, the fast equilibrium time and the possibility of using new selectors, since only small amounts are required. Determination of enantiomeric excess is of special importance in a number of situations. Example is to evaluate the purity of chiral synthetic building blocks and chiral pharmaceuticals. Such impurities originate from the synthesis or may be a consequence of poor configurational stability. In many cases, determination of unwanted enantiomer impurity (distomer) in the presence of the active enantiomer drug (eutomer) is needed. According to the ICH guideline detection limits of 0.1% impurities relative to the main compound are widely accepted as a minimum requirement for chiral trace impurity determination.

Chiral separations are among the most widely used applications of CE. Indirect methods are based on the formation of stable diastereoisomers with a chiral derivatization reagent. These diastereoisomers can then be separated, based upon their different physicochemical properties, using an achiral BGE. However, there are several drawbacks to this method. A suitable, nearly 100% pure enantiomeric derivatization reagent is needed [64], the derivatization procedure is time-consuming and reacting groups (amino, carboxyl) are requested [65]. Furthermore, racemization can take place during the derivatization process. The most widely used method is the direct chiral determination using chiral selectors added directly to the BGE. Chiral selectors include bile salts and some antibiotics as streptomycin but the most widely used ones are cyclodextrins [66-68].

1.2.3.3 Cyclodextrins as chiral selectors

The use of CDs in CZE mode is by far the most popular means of obtaining chiral separation in CE. Cyclodextrins are natural and neutral occurring cyclic oligosaccharides composed of several glucopyranose units. They have a bucket-like shape with a cavity of different dimensions depending on the cyclodextrin type (the number of glucose units and the substituent groups). Their cavity is relatively hydrophobic and able to accept guest compounds of different types, particularly those with non-polar groups. The outside rim of the cyclodextrin is relatively hydrophilic due to the presence of hydroxyl groups (primary and secondary). Figure 15 gives the structure of native cyclodextrins. Analyte molecules can become included into the core of the cyclodextrin through their complexation. The hydroxyl groups in the rim of the cyclodextrin can enantioselectively interact with a chiral analyte during its migration along the capillary. The two types of interaction between enantiomer and cyclodextrin are presented schematically in Figure 16 [69].

Drug enantiomers can fit inside the cavity of CD and have different interactions and individual binding constants. Thus, it is possible to chirally resolve them using cyclodextrin added into the CE electrolyte. There is a range of a native and derivatized cyclodextrins commercially available. The native CDs, α, β, and γ possess six, seven and eight glucose subunits, respectively. From theoretical point of view, the α -CD is best selected when the analyte does not contain an aromatic ring or contains one ring without ortho or meta substitution. The β-CD is best selected when the analyte contains one aromatic ring with ortho or meta substitution or two aromatic rings. The γ-CD is best selected when the analyte contains more than two aromatic rings [33]. However, in practice these rules may not apply as other factors and the selected separation conditions also control the success of separation. The surface hydroxyl group of the native cyclodextrin can be chemically replaced with various groups giving different types of derivatized uncharged and charged cyclodextrins (Table 4).



R1,R2, and R3 are possible positions for the derivatization of cyclodextrins

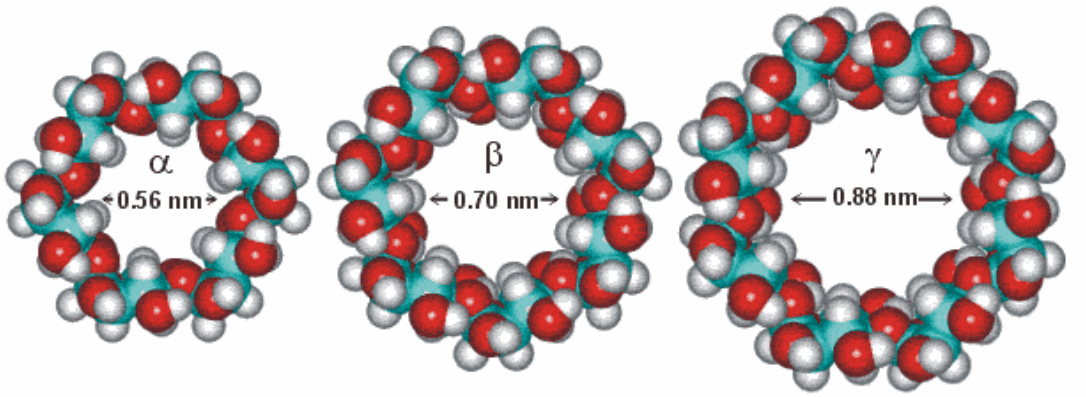


Figure 15: Structure of the native cyclodextrins.

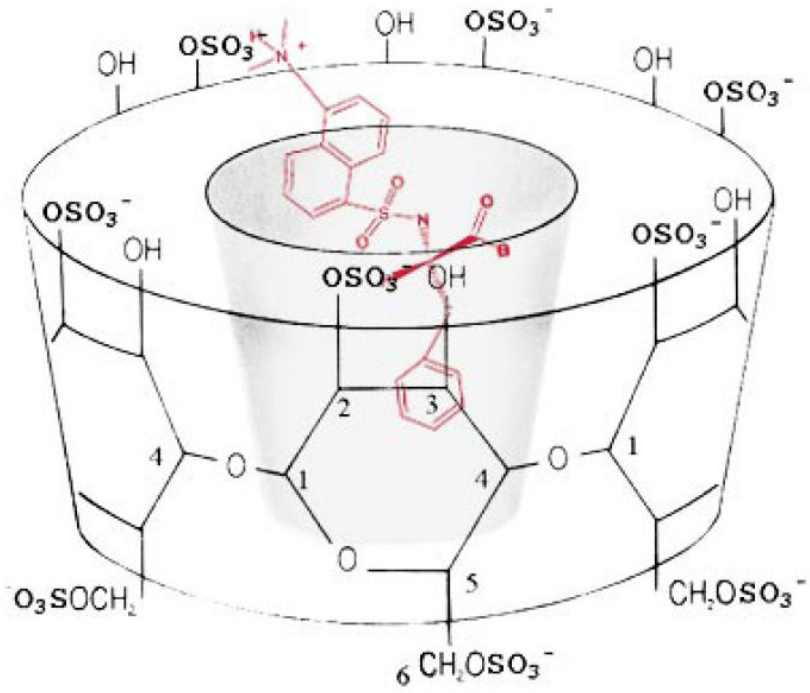


Figure 16: Proposed mechanism of interaction between DNS-Phenylalanine and HS-γ-CD. A key interaction point for chiral recognition appears to be at the C3 secondary hydroxyl.

Table 4: Native and derivatized cyclodextrins.

Cyclodextrin	Type
α-Cyclodextrin	Native cyclodextrin
β-Cyclodextrin	Native cyclodextrin
γ-Cyclodextrin	Native cyclodextrin
Hydroxypropyl-α-cyclodextrin	Derivatized uncharged cyclodextrin
Hydroxypropyl-β-cyclodextrin	Derivatized uncharged cyclodextrin
Hydroxypropyl-γ-cyclodextrin	Derivatized uncharged cyclodextrin
Highsulfated-α-cyclodextrin	Derivatized negatively charged cyclodextrin
Highsulfated-β-cyclodextrin	Derivatized negatively charged cyclodextrin
Highsulfated-γ-cyclodextrin	Derivatized negatively charged cyclodextrin
Phosphated-α-cyclodextrin	Derivatized negatively charged cyclodextrin
Phosphated-β-cyclodextrin	Derivatized negatively charged cyclodextrin
Phosphated-γ-cyclodextrin	Derivatized negatively charged cyclodextrin
Succinylated-β-cyclodextrin	Derivatized negatively charged cyclodextrin
Quaternary ammonium cyclodextrin	Derivatized positively charged cyclodextrin

These derivatized CDs have different enantioselectivities than the native CDs and are generally more water soluble than the naturally occurring ones.

Charged CDs offer the possibility of separating neutral drug enantiomers or enhancing the separation of ionic drugs. Figure 17 shows the structure of a cyclodextrin that has been chemically substituted with sulphate groups. These sulphated cyclodextrins have additional interactions with the analytes and therefore offer different separation possibilities.

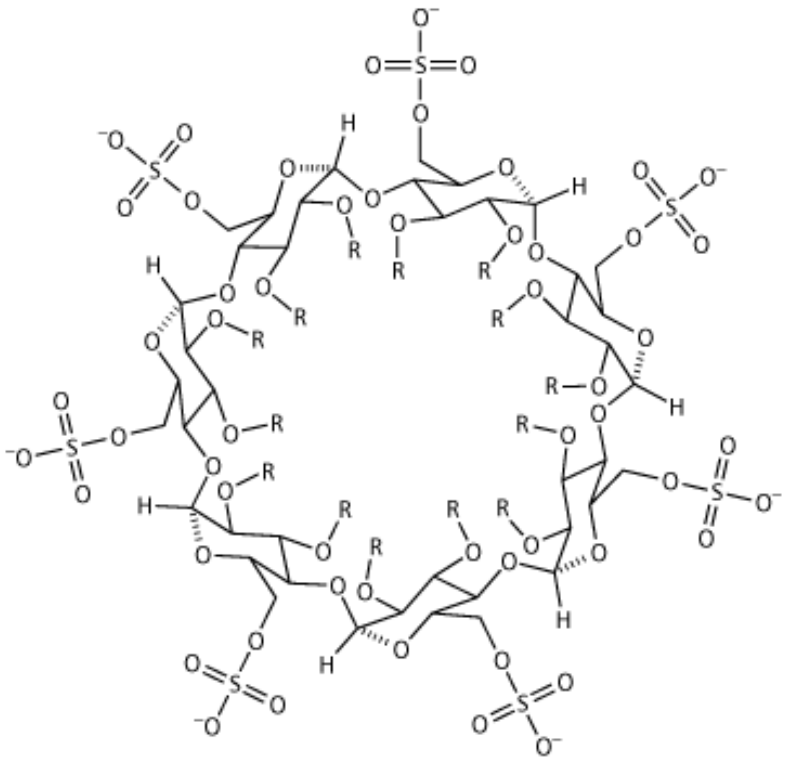


Figure 17: Chemical structure of highly sulphated β-cyclodextrin, R = (-SO_3^-) or (-H).

1.2.3.4 Advantages of CE over HPLC in chiral separation

In CE different separation modes as CZE and MEKC can be used and there is no need for expensive special accessories as the chiral HPLC column because the chiral selector is simply added to the background electrolyte (BGE). Moreover different new chiral selectors could be tried or a combination of two in a dual system. Also smaller volumes of buffer and sample are used. The reproducibility should in

principle be better than in HPLC as the chiral selector is replenished after each run. In addition higher efficiency and resolution can be obtained using CE for chiral separation. However, because of the low injection volume, the concentration sensitivity is low and stacking procedures are therefore sometimes needed. In addition, precision of injection is worse compared to HPLC. In the latter a known amount of sample is injected on column, which is not the case for CE due to the different injection mechanisms.

2. Materials and Methods

2.1 Experimental HPLC part

2.1.1 Chemicals and reagents

Acetonitrile HPLC grade, pilocarpine hydrochloride 99% and propranolol hydrochloride 99% were purchased from Acros Organics (Belgium). Methanol HPLC grade was purchased from Fisher Scientific (United Kingdom). Phosphoric acid 85%, triethylamine, sulfuric acid, sodium lauryl sulphate, ammonia, lactose monohydrate, polyvidone 25000, microcrystalline cellulose and magnesium stearate were purchased from Merck (Germany). Human insulin synthetic 95-98% (HPLC) approx. 24 IU/mg and tetrabutylammonium dihydrogen phosphat were purchased from Sigma-Aldrich (Switzerland). The following substances 1-[[4-[2-[(5- chloro-2-methoxy-benzoyl) amino]ethyl]phenyl] sulphonyl]-3-cyclohexylurea (glibenclamide) (purity >99.9%), 5-chloro-2-methoxy-*N*-[2-(4-sulfamoyl-phenyl)ethyl]benzamide (related compound A) (99.0%), methyl[[4-[2-[(5-chloro-2-methoxybenzoyl)amino]ethyl]phenyl]-sulfonyl]carbamate] (related compound B) (95.6%), 1-[[4-[2-[(3-ethyl-4-methyl-2-oxo-2,5dihydro-1*H*-pyrrol-1-yl)carbonyl]-amino]ethyl]phenyl]sulphonyl]-3-(*trans*-4methyl-cyclohexyl) urea (glimepiride) (99.7%) and Insuman® Basal 100 IU/ml suspension for injection were provided by Aventis (Germany). Anhydrous sodium sulphate was purchased from Lenziug (Australia). Potassium dihydrogen phosphate, sodium dihydrogen phosphate dihydrate, disodium hydrogen phosphate, and phosphoric acid 85% were purchased from Riedel-de-Haën (Germany). Sodium starch glycolate and ferric oxide were purchased from Caelo (Germany), respectively. Nicotinic acid, resorcin, phenol, salicylic acid, benzoic acid, 4-hydroxy benzoic acid and 2-naphthol, aniline, N-methylaniline, N-ethylaniline, 4-ethylaniline, dimethyaniline, N,N-diethyaniline, codeine phosphate, ephedrine hydrochloride, theophiline ethyendiamine, atropine sulfate, yohimbine hydrochloride, papaverine hydrochloride and butylscopolamine bromide (all were gifts from different pharmaceutical companies). Bi-distilled water was used throughout. All used chemicals were at least of analytical grade.

2.1.2 Instrumentation

Analyses were performed on a Merck Hitachi HPLC system, consisting of a solvent pump (model L 6200 A), an autosampler (AS 2000A), a UV-VIS detector (L-4250), a diode array detector (L-7450), and an interface (D-6000). The column oven (T1) was

from Techlab (Erkerode, Germany). The data were collected and analyzed using the D7000 HSM software (Merck). The separation was performed on a Superspher 100 RP-18e column (endcapped, 4 µm particle size, 125 mm × 4 mm, Merck) and a set of seven Chromolith Performance RP-18e (100 × 4.6 mm, Merck).

2.1.3 Separation conditions

2.1.3.1 Pilocarpine

2.1.3.1.1 Chromatographic conditions

The mobile phase was prepared according to a previously recommended method [70] by mixing 980 ml buffer solution with 20 ml methanol (The buffer was prepared by mixing 13.5 ml of 85% phosphoric acid, 3 ml of triethylamine and water to a total volume of 1000 ml. The pH was adjusted to 3 by the addition of 50% sodium hydroxide). The mobile phase was degassed by sonication before use. The flow rate was 1ml/min on the conventional column, while different flow rates from 1 to 9 ml/min were applied on monolithic columns. The injection volume was 20 µl and the detection wavelength was 214 nm. All separations were performed at ambient temperature.

2.1.3.1.2 Preparation of standard

The buffer described in section 2.1.3.1.1 was used as sample diluent. Dilutions were carried out using the sample diluent, to obtain solutions of known concentrations to be used for the standard preparation and the assay purposes. The concentration levels described in European Pharmacopoeia 2005 (0.008-0.5 mg/ml) were used.

2.1.3.1.3 Preparation of degradation products

Solution a (Isopilocarpine): Isopilocarpine was obtained from pilocarpine. 1 ml of 0.1 *M* sodium hydroxide solution was added to a 5 ml solution of 0.5 mg/ml pilocarpine hydrochloride in a 25 ml volumetric flask to allow deprotonation followed by the addition of 1 ml 0.1 M HCl to allow reprotonation. The volume of the finally resulting solution was completed to 25 ml by sample diluents.

Solution b (Pilocarpic acid & isopilocarpic acid): Pilocarpic acid and isopilocarpic acid which are not commercially available were generated by base catalyzed hydrolysis in a way similar to [71]. To 5 ml of 1 mg/ml pilocarpine aqueous solution in a 25 ml volumetric flask, 100 µl of concentrated ammonia was added and the mixture was heated in an oven to 90 ºC for about 2 hours. The mixture was allowed to cool to room temperature and then diluted to 25 ml with sample diluent.

Pilocarpine/degradation products mixture: The final mixture that contains pilocarpine with its three degradation products was prepared by mixing 8 ml of solution a, 8 ml of solution b and 5 ml of 0.5 mg/ml pilocarpine hydrochloride solution. The solution was completed to a total volume of 25 ml using sample diluent.

2.1.3.1.4 Addition of excipient

Excipient for pilocarpine hydrochloride ophthalmic solution was prepared containing the inactive ingredients disodium edetate 4 mg, polyvinylpyrrolidone 1.7 mg, sodium dihydrogen phosphate 0.8 mg, disodium hydrogen phosphate 0.94 mg, sodium chloride 0.9 mg and benzalkonium chloride 0.13 mg in 100 ml of bi-distilled water.

2.1.3.2 Propranolol

2.1.3.2.1 Chromatographic conditions

The mobile phase was prepared according to the method described in the European Pharmacopoeia 1997, by mixing 1.15 g sodium lauryl sulphate, 10 ml of a mixture of 1 volume of sulfuric acid and 9 volumes of water, 20 ml of 17 g/l solution of tetrabutylammonium dihydrogen phosphate, 370 ml of water and 600 ml of acetonitrile. The pH of the finally resulting solution was corrected to 3.3 using diluted sodium hydroxide solution. The flow rate was 1 ml/min on the conventional column, while different flow rates (1 to 9 ml/min) were applied on monolithic column. The injection volume was 20 µl and the detection wavelength was 292 nm. All separations were performed at ambient temperature.

2.1.3.2.2 Preparation of standard

The primary stock solution of propranolol hydrochloride was prepared in the mobile phase to obtain solutions of known concentrations to be used for the standard preparation and the assay purposes in the range of 0.002 - 1 mg/ml.

2.1.3.2.3 Preparation of degradation products

The two main degradation products of propranolol hydrochloride are 3-(Naphthalene-1-yloxy)propane-1,2-diol and 1,1'-[(1-Methylethyl)imino]bis[3-(naphthaline-1-yloxy)-pro- pane-2-ol]. They were generated by basic hydrolysis by the addition of a 1 ml of 0.1 *M* NaOH to a 5 ml solution of 1 mg/ml propranolol hydrochloride in a 25 ml volumetric flask. The solution was left for 20 min to allow hydrolysis. Then 1 ml 0.1 *M* hydrochloric acid was added to neutralize the solution. The volume of the finally resulting solution was completed to 25 ml with mobile phase.

2.1.3.2.4 Addition of excipient

Excipient for propranolol hydrochloride tablets was prepared containing the following substances specified as a percentage of tablet weight, propranolol hydrochloride 26.7% w/w, lactose monohydrate 51.3% w/w, microcrystalline cellulose 20% w/w, and magnesium stearate 2% w/w [72].

2.1.3.3 Glibenclamide and glimepiride

2.1.3.3.1 Chromatographic conditions

The mobile phase was prepared by dissolving 650 mg sodium dihydrogen phosphate dihydrate in 550 g water and adding two drops of phosphoric acid 85% and 351.5 g acetonitrile to 1000 ml. The pH of the resulting solution before the addition of acetonitrile was in the range of 3.0 - 3.3. The flow rate was 1.25 ml/min on the conventional column, while different flow rates (1.25 to 9 ml/min) were applied on monolithic columns. The injection volume was 10 µl and the detection wavelength for glibenclamide, related compounds A and B was 210 nm and for glimepiride was 228 nm. The column oven temperature was set at 35˚C for all runs.

2.1.3.3.2 Preparation of sample solvent

The sample solvent consisted of 20 volumes of 4 m*M* phosphate buffer (pH 7) and 80 volumes acetonitrile. The buffer was prepared based on British Pharmacopoeia 1999 [73] by mixing 21.2 mg potassium dihydrogen phosphate and 34.65 mg sodium dihydrogen phosphate dihydrate in 100 ml water.

2.1.3.3.3 Preparation of standard

A synthetic mixture of drug product compounds was prepared containing glibenclamide, glimepiride and the related products A and B at different concentrations in the range of 0.001 - 0.240 mg/ml.

2.1.3.3.4 Addition of excipient

Inactive ingredients for glibenclamide and glimepiride tablets were prepared containing the following substances: 74.6 mg lactose monohydrate, 4.0 mg sodium starch glycolate, 0.5 mg polyvidone 25000, 10.0 mg microcrystalline cellulose, 0.5 mg magnesium stearate and 0.4 mg ferric oxide. The mixture was homogenized by trituration in a mortar.

2.1.3.4 Insulin

2.1.3.4.1 Chromatographic conditions

The mobile phase was prepared by dissolving 28.4 g anhydrous sodium sulphate in 1000 ml water followed by the addition of 2.7 ml of concentrated phosphoric acid 85% (buffer pH 2.3). For the conventional column 74 volumes of the buffer were mixed with 26 volumes of acetonitrile. For monolithic columns, 74.5 volumes of the buffer were mixed with 25.5 volumes of acetonitrile. The mobile phase was degassed by sonication before use. The flow rate was 1 ml/min on the conventional column, while different flow rates from 1 to 9 ml/min were applied on monolithic columns. The injection volume was 10 µl and the detection wavelength was 214 nm. All separations were performed at ambient temperature.

2.1.3.4.2 Preparation of standard

Standard solutions of human insulin were prepared in 0.9% w/v sodium chloride solution with the addition of 5 drops of 0.1 *M* hydrochloric acid to improve solubility. Solutions of known concentrations to be used for the standard preparation and the assay purposes were prepared in the range of 0.03 to 3.00 mg/ml.

2.1.3.4.3 Preparation of mixture

Pharmaceutical preparation that contains human insulin commercially referred to as Insuman Basal^{TM} 100 IU/ml (suspension for injection) was used. The other ingredients of Insuman Basal^{TM} are: protamine sulphate, m-cresol, phenol, zinc chloride, sodium dihydrogen phosphate dihydrate, glycerol, sodium hydroxide and hydrochloric acid.

2.1.3.5 Acid mixture

2.1.3.5.1 Chromatographic conditions

The used column was Chromolith Performance RP-18e (100 × 4.6 mm, Merck). The different pH buffers used during the method development were prepared according to Table 5. A mixture of methanol: 25 m*M* phosphate buffer pH 3 (10:90) was finally selected during method development as mobile phase. The flow program mentioned in Table 23 section 3.1.3.1.1 was finally selected. Detection wavelength was 273 nm.

2.1.3.5.2 Preparation of standard

A mixture of seven acidic compounds namely: nicotinic acid, resorcin, phenol, salicylic acid, benzoic acid, 4-hydroxy-benzoic acid and 2-naphthol, has been created

by weighing 2 mg of each compound in a 10 ml volumetric flask and filling with water to the mark.

2.1.3.6 Aniline and its derivatives mixture

2.1.3.6.1 Chromatographic conditions

The used column was Chromolith Performance RP-18e (100 × 4.6 mm, Merck).

A mixture of phosphate buffer pH 5, 25 m*M*: methanol (55:45) was finally selected during method development as mobile phase. The flow program mentioned in Table 24 section 3.1.3.1.2 was finally selected. All compounds were detected at 214 nm.

2.1.3.6.2 Preparation of standard mixture

A mixture of six basic compounds consisting of aniline and five derivatives, namely: N-methylaniline, N-ethylaniline, 4-ethylaniline, dimethylaniline and N,N-diethylaniline, has been created by weighing 2 mg of each compound in a 10 ml volumetric flask and filling to the mark with the mobile phase which was a mixture of phosphate buffer pH 5, 25 m*M*:methanol (55:45).

Table 5: Buffer preparations.

Standard solutions used for the preparation of 2 l buffer solution	**pH 2**	**pH 3**	**pH 4**	**pH 5**	**pH 6**	**pH 7**	**pH 7.4**	**pH 8**
0.1*M* Phosphoric acid 6.86 ml/l	282.5	55.0	-	-	-	-	-	-
0.1*M* Acetic acid 5.8 ml/l	-	-	410.0	148.0	-	-	-	-
0.1*M* Sodium acetate solution 8.2 g/l	-	-	90.0	352.0	-	-	-	-
0.1*M* Sodium dihydrogen-phosphate dihydrate solution 15.6 g/l	217.5	445.0	-	-	438.5	195.0	95.0	26.5
0.1*M* Disodium hydrogenphosphate dihydrate solution 17.8g/l	-	-	-	-	61.5	305.0	405.0	473.5

2.1.3.7 Alkaloid mixture

2.1.3.7.1 Chromatographic conditions

The used column was Chromolith Performance RP-18e (100 × 4.6 mm, Merck).

A mixture of phosphate buffer 25 m*M*, pH 3: methanol (80:20) was finally selected during method development as mobile phase. The flow program mentioned in Table 25 section 3.1.3.1.3 was finally selected. All compounds were detected at 214 nm.

2.1.3.7.2 Preparation of standard mixture

A basic mixture of seven alkaloids has been created by putting 2 mg of each in a 10 ml volumetric flask and filling to the mark with the mobile phase which was a mixture of phosphate buffer 25 m*M*, pH 3: methanol (80:20). The compounds were namely: codeine phosphate, ephedrine hydrochloride, theophiline ethylendiamine, atropine sulfate, yohimbine hydrochloride, papaverine hydrochloride and butylscopolamine bromide with two of its impurities butylscopolamine impurity a and butylscopolamine impurity b.

2.2 Experimental CE

2.2.1 Chemicals and reagent

The following racemic drugs were analysed: atenolol hydrochloride, alprenolol hydrochloride, ephedrine hydrochloride, methadone hydrochloride, pindolol hydrochloride, promethazine hydrochloride, propranolol hydrochloride, tryptophan and verapamil hydrochloride (all gifts from different pharmaceutical companies).

The cyclodextrin selectors used: α-cyclodextrin, β-cyclodextrin, γ-cyclodextrin, hydroxypropyl-α-cyclodextrin, hydroxypropyl-β-cyclodextrin, hydroxypropyl-γ-cyclodextrin, carboxymethyl-β-cyclodextrin and high sulphated-β-cyclodextrin were all purchased from Sigma-Aldrich (Germany). Succinyl-β-cyclodextrin was purchased from Sigma-Aldrich (Hungary). The α-cyclodextrin phosphate sodium salt and γ-cyclodextrin phosphate sodium salt were purchased from Sigma-Aldrich (Switzerland). High sulfated-α-cyclodextrin and high sulfated-γ-CD was comercialized as 20% (w/v) aqueous solutions from Beckman-Coulter (Fullerton, CA).

2.2.2 Instrumentation

Screening of racemic drugs were carried out on a UniCAM Crystal 310 CE System (UniCAM Ltd., Cambridge, United Kingdom) equipped with a Spectra Physics 100 UV detector. Bare fused silica capillaries were obtained from (Polymicro Technologies,

Phoenix, AZ, United State of America); 50 µm (i.d.), 62 cm total length (L_t), L_d = 48 cm to the detector window. A second instrument with different instrumental parameters was also used: A PrinCE 550 CE System (Prince Technologies, Emmen, Netherlands) equipped with a Lambda 1010 UV detector (Bischoff, Leonberg, Germany) and the bare fused silica capillary with a total length (L_t) amounted to 85 cm, the length to the detection window (L_d) amounted to = 31 cm.

pH measurements were performed on a Metrohm 620 pH meter (Herisau, Switzerland). Rotilabo® - syringe filters were obtained from Carl Roth (PVDF, 0.22 µm, Karlsruhe, Germany).

2.2.3 Separation conditions

2.2.3.1 Preparation of racemic solutions

Racemic drugs were dissolved in the 5 m*M* phosphate buffer pH 2.5 (10 folds dilution of the running buffer) at a concentration of 0.2 mg/mL. All sample solutions were filtered through the 0.22 µm Rotilabo® - syringe filters obtained from Carl Roth (PVDF, Karlsruhe, Germany).

2.2.3.2 Preparation of separation buffer

A 50 m*M* phosphate buffer, pH 2.5 was prepared by dissolving 6.89 g sodium dihydrogen phosphate dihydrate in 1000 ml volumetric flask with bi-distilled water and adjusting the pH to 2.5 using 0.1 *M* perchloric acid.

2.2.3.3 Cyclodextrin preparation

Each of the cyclodextrin 2% solutions was prepared in the 50 m*M* phosphate buffer pH 2.5 by dissolving 40 mg CD in 2 ml of the phosphate buffer pH 2.5. For HS-α-CD and HS-γ-CD which were commercialized as 20% (w/v) aqueous solutions, the 2% solution was prepared by mixing 200 µl of the 20% solution with 1800 µl phosphate buffer pH 2.5.

2.2.3.4 Electrophoretic conditions

Pressure injections (50 mbar for 0.3 min) of standard sample solutions (racemic drug) were used. Complete filling technique of the capillary with cyclodextrin has been used. This technique involves filling the capillary and doing the run from a solution of CD in the 50 m*M* phosphate buffer pH 2.5 electrolytes. Partial filling technique, which involves filling the capillary from a solution of CD in the buffer, then doing the run from the buffer solution without CD was not successful (did not lead to any separation). The running buffer contained 2% of any of the CD. The voltages were either 25 kV or -25

kV depending on the separation mode (normal or reversed). For negatively charged cyclodextrins the reversed separation mode was found to be more effective for basic drug enantiomeric separation. Temperature of the capillary was set to 25°C. Detection was carried out at 200 nm for all compounds. The capillaries were conditioned before use by successive washings (under a pressure of 1500 mbar) for 30 min with 0.1 *M* sodium hydroxide then 30 min water, followed by a 30 min flush with run buffer.

3 Results

3.1 Results HPLC part

3.1.1 Transferred methods

3.1.1.1 Optimization of chromatographic parameters

In order to evaluate the performance of monolithic columns it was necessary to compare with that of particle packed columns. HPLC methods previously described for the determination of pilocarpine, propranolol, glibenclamide, glimepiride and insulin using conventional particle-packed C18 columns have been adapted to our conventional column Superspher 100 RP-18e column (endcapped, 4 µm particle size, 125 mm × 4 mm, Merck). Structures of pilocarpine, propranolol, glibenclamide, insulin each with its related compounds are shown in Figures 18, 19, 20 and 21, respectively. The methods were then transferred without further modification to the monolithic Chromolith Performance RP-18e (100 × 4.6 mm, Merck). For pilocarpine a method described in [71] was taken. The used method has been previously tested on a set of conventional C18 columns, however was not tested on Superspher commercial type column, which was used in this study. For propranolol the method used based on European Pharmacopoeia 1997 [74], however some parameters were changed including the flow rate and the properties of the octadecylsilyl silica column used. The conventional Superspher 100 RP-18e column (endcapped, 4 µm particle size, 125 mm × 4 mm, Merck) was used with a flow rate of 1 ml/min, instead of a 5 µm particle size, 200 mm × 5 mm octadecylsilyl silica column at a flow rate of 1.8 ml/min as specified in European Pharmacopoeia 1997. The conventional C18 based method used for glibenclamide, glimepiride and the two related substances was formerly developed in our laboratories [75]. For insulin the method was adapted from the European Pharmacopoeia 2005 [76], isocratic elution with a 26% acetonitrile concentration was chosen and the Superspher 12.5 cm column packed with 4 µm particles instead of 25 cm column packed with 5 µm particles specified in the European Pharmacopoeia 5th edition.

3.1.1.2 Method transfer and validation

Methods for pilocarpine, propranolol, glibenclamide and glimepiride were found to be successfully transferable from the conventional particle-packed to the monolithic rod columns without any modification. For the relatively larger molecule insulin, the method was not successfully transferred from the first go.

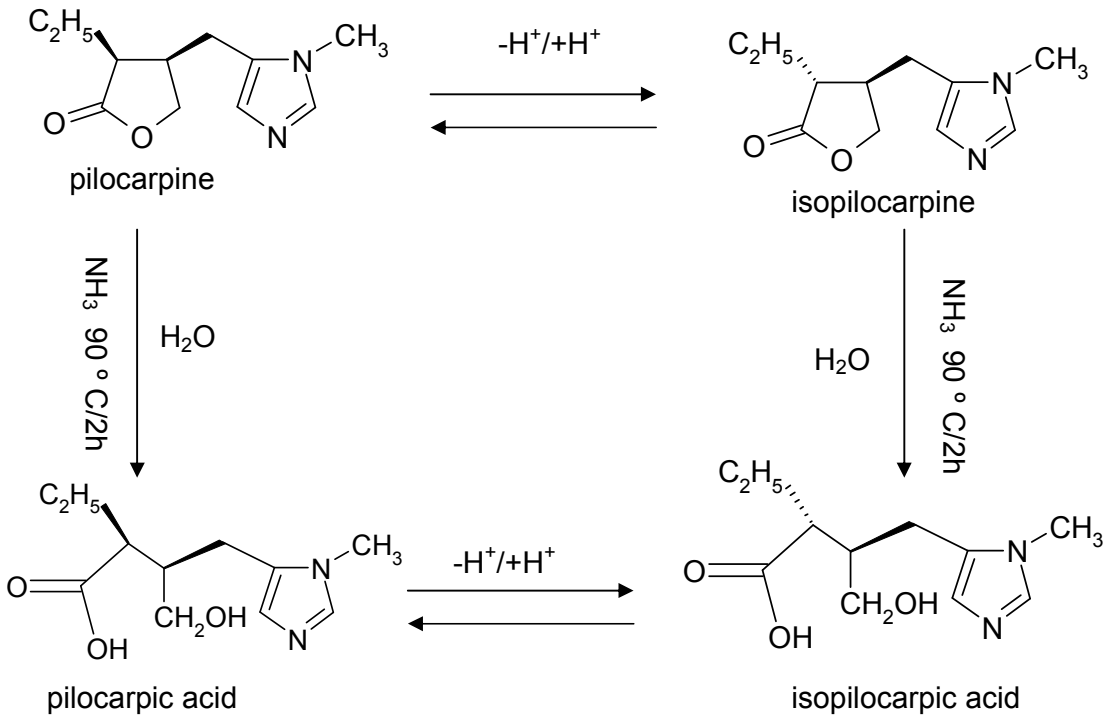


Figure 18: Degradation scheme for pilocarpine.

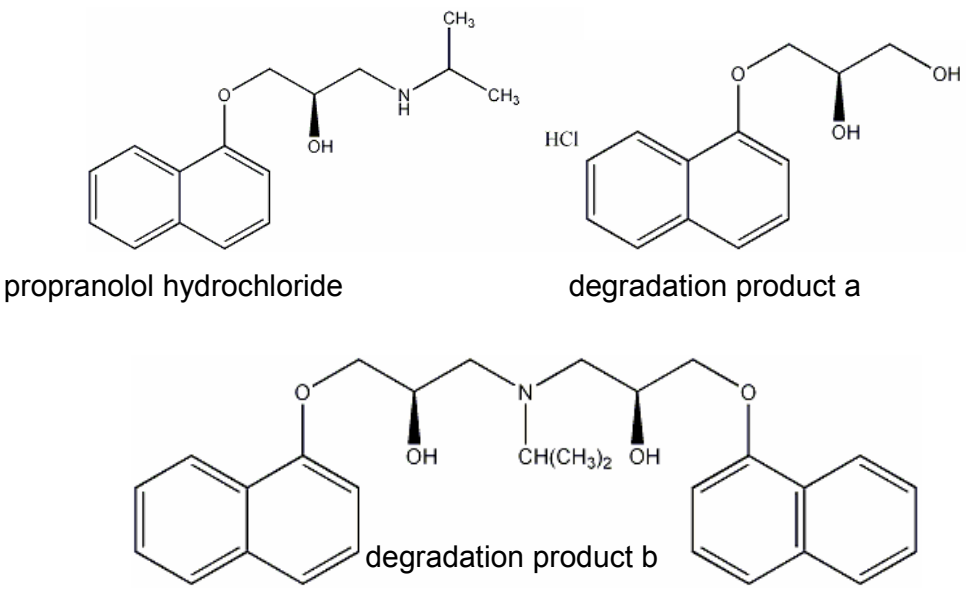


Figure 19: Structure of propranolol hydrochloride and its two degradation products.

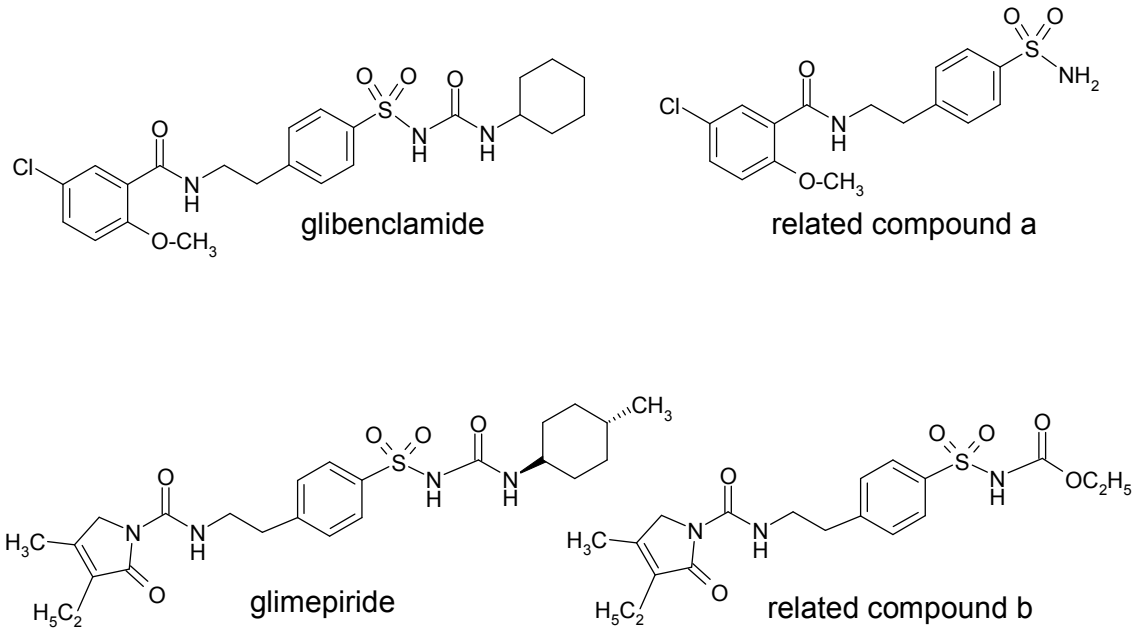


Figure 20: Structure of glibenclamide and its related substances.

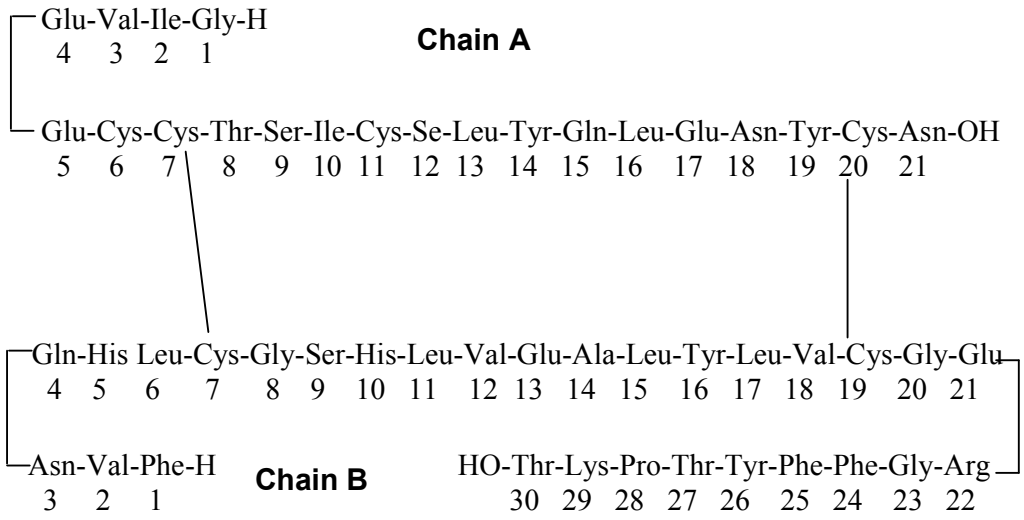


Figure 21: Structure of human insulin.

The peak of insulin was eluted too early when the method was firstly transferred to the monolithic column, so it overlapped with the peak of m-cresol (Figure 22). However, by decreasing the amount of the organic modifier (acetonitrile) in the mobile phase from 26% to 25.5% a good resolution was obtained (results will be shown later in Figures 23-26). It has been demonstrated, that the same elution order and pattern of the used mixtures were obtained in monolithic and conventional columns. This indicates that the selectivity of the two column types is equivalent due to the very similar chemical properties of the two column types.

3.1.1.2.1 Specificity

The specificity of the methods was examined by observing if there was any interference of the inactive ingredients of the pharmaceutical preparations in each drug case. The HPLC chromatograms recorded for the inactive ingredients of the analyzed compounds showed no peaks at the retention times of the active drugs and their degradation products or related compounds.

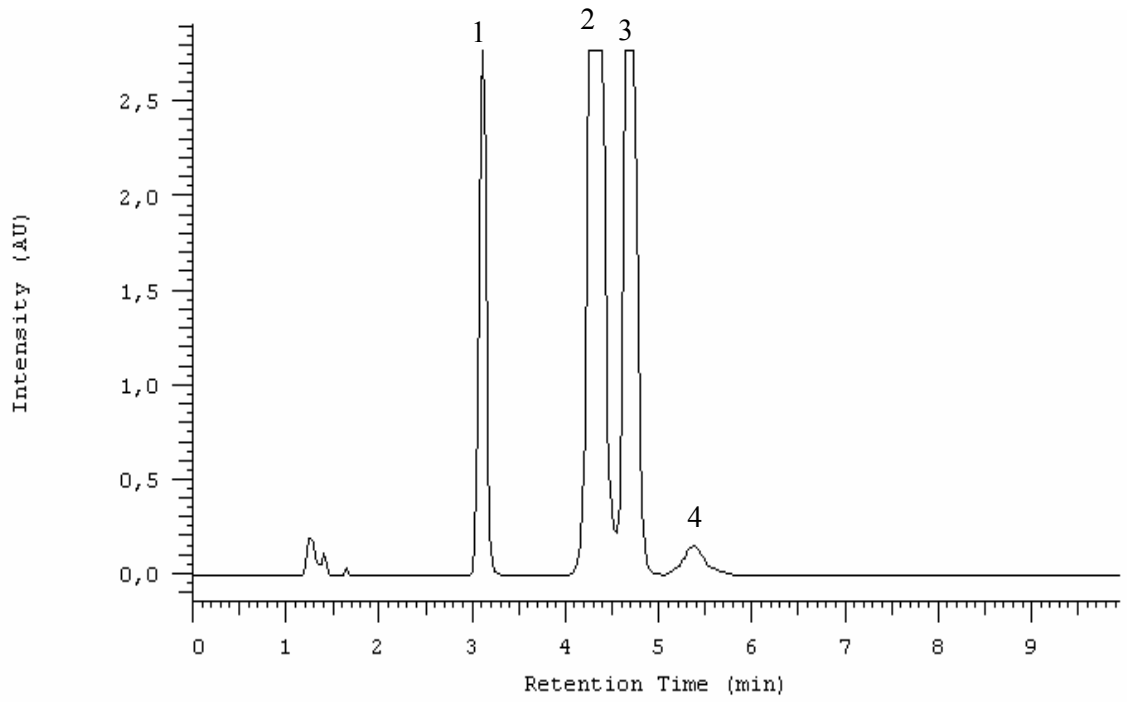


Figure 22: Representative chromatogram for phenol (1), m-cresol (2), human insulin (3) and 21-desamido human insulin (4) on monolithic (Chromolith Performance RP-18e) column at a flow rate of 1ml/min. Mobile phase consists of buffer pH = 2.3: acetonitrile (74:26, v/v).

The specificity was also demonstrated by the good separation of the degradation products and/or related compounds from the main compound peak in each method.

3.1.1.2.2 Accuracy

The accuracy of the methods was tested by determination of the recovery using the excipient used in drug formulation of each of the used drugs. Recovery values are shown in Table 6. Results indicate a good recovery for all of the tested compounds.

Table 6: List of recovery results for pilocarpine, propranolol, glibenclamide and glimepiride at three concentration levels.

Drug	Column type	Theoretical value (mg/ml)	Mean recovery* (mg/ml)	Recovery (%)	RSD (%)
pilocarpine hydrochloride	Superspher RP-18e	0.008	0.0076	95.36	0.84
		0.200	0.1920	96.00	0.65
		0.500	0.4960	99.20	0.70
	Chromolith Performance RP-18e	0.008	0.0077	96.50	0.81
		0.200	0.1960	98.23	0.66
		0.500	0.4990	99.80	0.30
propranolol hydrochloride	Superspher RP-18e	0.020	0.0197	98.95	1.97
		0.040	0.0395	98.00	1.26
		0.080	0.0794	99.27	0.91
	Chromolith Performance RP-18e	0.020	0.0198	99.00	1.91
		0.040	0.0398	99.5 0	1.23
		0.080	0.0796	99.60	1.00
glibenclamide	Superspher RP-18e	0.160	0.1596	99.80	0.69
		0.200	0.2001	100.05	1.27
		0.240	0.2393	99.71	0.91
	Chromolith Performance RP-18e	0.160	0.1598	99.92	0.56
		0.200	0.1996	99.83	0.70
		0.240	0.2402	100.08	0.69
glimepiride	Superspher RP-18e	0.160	0.1612	100.75	0.86
		0.200	0.1976	98.80	0.91
		0.240	0.2397	99.87	1.13
	Chromolith Performance RP-18e	0.160	0.1610	100.62	1.02
		0.200	0.1994	99.70	0.86
		0.240	0.2405	100.20	0.95

* Mean value of 10 determinations.

3.1.1.2.3 Precision

Precision which was an important topic of this study was carefully tested. To ensure assay precision within day (5 injections were performed each day) and between days

(determined at 5 days) precisions were assessed at 3 concentration levels on the conventional (Superspher) and the monolithic columns for each separation. Precision results for pilocarpine, propranolol, glibenclamide, and insulin are summarized in Tables 7-a and b, 8-a and b, 9-a and b and 10-a and b, respectively.

Table 7-a: Within day repeatabilities of pilocarpine hydrochloride on conventional and monolithic columns over a concentration range 0.008 - 0.500 mg/ml using n = 5.*

Column type and No.		Within day repeatability RSD % of AUC			Within day repeatability RSD % of t_R
		0.008 mg/ml	0.200 mg/ml	0.500 mg/ml	(n = 15)
Superspher 100 RP-18e column		0.84	0.70	0.88	0.52
Chromolith Performance RP-18e Batch No. Um 1042 Rod No. 1042/020	1 ml/min	0.85	0.60	0.54	0.45
	4 ml/min	0.70	0.52	0.40	0.66
Chromolith Performance RP-18e Batch No. Um 1043 Rod No. 1043/041	1 ml/min	0.50	0.77	0.44	0.44
	4 ml/min	0.69	0.49	0.68	0.49
Chromolith Performance RP-18e Batch No. Um 1045 Rod No. 1045/036	1 ml/min	0.68	0.51	0.34	0.42
	4 ml/min	0.74	0.77	0.87	0.45
Chromolith Performance RP-18e Batch No. Um 1042 Rod No. 1042/012	1 ml/min	0.61	0.73	0.75	0.52
	4 ml/min	0.64	0.47	0.31	0.38
Chromolith Performance RP-18e Batch No. Um 1043 Rod No. 1043/032	1 ml/min	0.66	0.54	0.61	0.38
	4 ml/min	0.67	0.68	0.67	0.45
Chromolith Performance RP-18e Batch No. Um 1045 Rod No. 1045/023	1 ml/min	0.73	0.69	0.44	0.51
	4ml/min	0.58	0.62	0.49	0.48

* Results on monolithic columns include repeatabilities at flow rates of 1 and 4 ml/min

Table 7-b: Between days repeatabilities for pilocarpine hydrochloride on conventional and monolithic columns over a concentration range of 0.008 - 0.500 mg/ml using n = 5. *

Column type and No.		Between day repeatability RSD % of AUC			Between day repeatability RSD % of t_R
		0.008 mg/ml	0.200 mg/ml	0.500 mg/ml	(n = 15)
Superspher 100 RP-18e column		1.16	0.90	0.84	0.66
Chromolith Performance RP-18e Batch No. Um 1042 Rod No. 1042/020	1 ml/min	0.87	0.67	0.84	0.65
	4 ml/min	0.85	0.76	0.87	0.65
Chromolith Performance RP-18e Batch No. Um 1043 Rod No. 1043/041	1 ml/min	0.51	0.72	0.40	0.53
	4 ml/min	0.59	0.81	0.89	0.52
Chromolith Performance RP-18e Batch No. Um 1045 Rod No. 1045/036	1 ml/min	0.73	0.53	0.38	0.42
	4 ml/min	0.88	0.43	0.85	0.54
Chromolith Performance RP-18e Batch No. Um 1042 Rod No. 1042/012	1 ml/min	0.53	0.54	0.79	0.51
	4 ml/min	0.62	0.74	0.70	0.51
Chromolith Performance RP-18e Batch No. Um 1043 Rod No. 1043/032	1 ml/min	0.69	0.78	0.87	0.58
	4 ml/min	0.57	0.66	0.71	0.53
Chromolith Performance RP-18e Batch No. Um 1045 Rod No. 1045/023	1 ml/min	0.73	0.58	0.53	0.68
	4 ml/min	0.50	0.80	0.71	0.76

* Results on monolithic columns include repeatabilities at flow rates of 1 and 4 ml/min

Table 8-a: Within day repeatability for propranolol hydrochloride on conventional and monolithic columns over the concentration range 0.002 – 1.000 mg/ml, using n = 5.*

Column type and No.		Within day repeatability RSD (%) of AUC			Within day repeatability RSD (%) of t_R
		0.002 mg/ml	0.500 mg/ml	1.000 mg/ml	(n = 15)
Superspher 100 RP-18e column		1.27	0.70	0.69	0.76
Chromolith Performance RP-18e Batch No. Um 1042 Rod No. 1042/020	1 ml/min	0.78	0.66	0.43	0.42
	4 ml/min	0.92	0.61	0.57	0.66
Chromolith Performance RP-18e Batch No. Um 1043 Rod No. 1043/041	1 ml/min	0.88	0.86	0.61	0.56
	4 ml/min	0.91	0.96	0.60	0.66
Chromolith Performance RP-18e Batch No. Um 1045 Rod No. 1045/036	1 ml/min	0.79	0.61	0.58	0.66
	4 ml/min	0.92	0.56	0.38	0.66

* Results on monolithic columns include repeatabilities at flow rates of 1 and 4 ml/min

Table 8-b: Between days repeatabilities for propranolol hydrochloride on conventional and monolithic columns over the concentration range 0.002 – 1.00 mg/ml, using n = 5.*

Column type and No.		Between day repeatability RSD (%) of AUC			Between day repeatability RSD (%) of t_R
		0.002 mg/ml	0.500 mg/ml	1.00 mg/ml	(n = 15)
Superspher 100 RP-18e column		1.17	1.01	0.97	0.85
Chromolith Performance RP-18e Batch No. Um 1042 Rod No. 1042/020	1 ml/min	0.96	0.96	0.81	0.79
	4 ml/min	0.99	0.87	0.90	0.72
Chromolith Performance RP-18e Batch No. Um 1043 Rod No. 1043/041	1 ml/min	0.94	0.85	0.70	0.82
	4 ml/min	0.95	1.00	0.89	0.52
Chromolith Performance RP-18e Batch No. Um 1045 Rod No. 1045/036	1 ml/min	0.89	0.96	0.74	0.90
	4 ml/min	0.96	0.68	0.71	0.71

*Results on monolithic columns include repeatabilities at flow rates of 1 and 4 ml/min.

Table 9-a: Within day repeatability for glibenclamide on conventional and monolithic columns over a concentration range 0.01 - 0.24 mg/ml using n = 5. *

Column type and No.		Within day repeatability RSD (%) of AUC			Within day repeatability RSD (%) of t_R
		0.01 mg/ml	0.12 mg/ml	0.24 mg/ml	(n = 15)
Superspher 100 RP-18e column Flow rate 1.25 ml/min		0.99	0.62	0.64	0.16
Chromolith Performance RP-18e Batch No. Um 1042 Rod No. 1042/020	1.25 ml/min	0.79	0.65	0.36	0.10
	4 ml/min	0.90	0.59	0.41	0.00
Chromolith Performance RP-18e Batch No. Um 1043 Rod No. 1043/041	1.25 ml/min	0.89	0.51	0.64	0.00
	4 ml/min	0.74	0.52	0.57	0.00
Chromolith Performance RP-18e Batch No. Um 1045 Rod No. 1045/036	1.25 ml/min	0.82	0.52	0.46	0.32
	4 ml/min	0.84	0.63	0.55	0.20

* Rresults on monolithic columns include repeatabilities at flow rates of 1.25 and 4 ml/min

Table 9-b: Between days repeatability for glibenclamide on conventional and monolithic columns over a concentration range 0.01 - 0.24 mg/ml using n = 5.*

Column type and No.		Between day repeatability RSD (%) of AUC			Between day repeatability RSD (%) of t_R
		0.01 mg/ml	0.12 mg/ml	0.24 mg/ml	(n = 15)
Superspher 100 RP-18e column Flow rate 1.25 ml/min		1.25	0.71	0.75	0.85
Chromolith Performance RP-18e Batch No. Um 1042 Rod No. 1042/020	1.25 ml/min	0.84	0.72	0.60	0.21
	4ml/min	0.93	0.68	0.61	0.27
Chromolith Performance RP-18e Batch No. Um 1043 Rod No. 1043/041	1.25 ml/min	0.82	0.62	0.68	0.21
	4ml/min	0.89	0.69	0.74	0.54
Chromolith Performance RP-18e Batch No. Um 1045 Rod No. 1045/036	1.25 ml/min	0.92	0.57	0.53	0.39
	4 ml/min	0.92	0.67	0.61	0.27

* Results on monolithic columns include repeatabilities at flow rates of 1.25 and 4 ml/min

Table 10-a: Within day repeatability for human insulin on conventional and monolithic columns over a concentration range 0.03 - 0.30 mg/ml using n = 5.*

Column type and No.		Within day repeatability RSD (%) of AUC			Within day repeatability RSD (%) of t_R
		0.03 mg/ml	0.30 mg/ml	3.00 mg/ml	(n = 15)
Superspher 100 RP-18e column		1.08	0.68	0.74	0.67
Chromolith Performance RP-18e Batch No. UM1042 Rod No. 1042/020	1 ml/min	0.84	0.61	0.73	0.54
	4 ml/min	0.96	0.78	0.50	0.58
Chromolith Performance RP-18e Batch No. UM1043 Rod No. 1043/041	1 ml/min	0.89	0.42	0.31	0.57
	4 ml/min	0.67	0.84	0.59	0.45
Chromolith Performance RP-18e Batch No. UM5020 Rod No. 5020/028	1 ml/min	0.87	0.57	0.45	0.35
	4 ml/min	0.75	0.55	0.62	0.43

*Results on monolithic columns include repeatabilities at flow rates of 1 and 4 ml/min.

Table 10-b: Between days repeatability human insulin on conventional and monolithic columns over a concentration range 0.03 - 0.30 mg/ml using n = 5.*

Column type and No.		Between day repeatability RSD (%) of AUC			Between day repeatability RSD (%) of t_R
		0.03 mg/ml	0.30 mg/ml	3.00 mg/ml	(n = 15)
Superspher 100 RP-18e column		1.20	0.96	0.90	0.98
Chromolith Performance RP-18e Batch No. UM1042 Rod No. 1042/020	1 ml/min	0.79	0.95	0.70	0.75
	4 ml/min	1.03	0.91	0.75	0.66
Chromolith Performance RP-18e Batch No. UM1043 Rod No. 1043/041	1 ml/min	0.97	0.75	0.87	0.70
	4 ml/min	0.93	0.73	0.93	0.80
Chromolith Performance RP-18e Batch No. UM5020 Rod No. 5020/028	1 ml/min	0.97	0.93	0.50	0.82
	4 ml/min	0.97	0.85	0.90	0.60

*Results on monolithic columns include repeatabilities at flow rates of 1 and 4 ml/min

Column to column reproducibility for Chromolith Performance was measured in each drug method. A set of six monolithic columns originating from three different batches were tested for the pilocarpine method. For the propranolol, glibenclamide and insulin methods, column to column reproducibility was tested using three monolithic columns from different batches. Results are summarized in Table 11. A total of seven monolithic columns from 4 different batches were used in this study.

Table 11: Monolithic column to column reproducibility calculated for each method, 7 different columns from 4 different batches were used in this estimation.

Drug method	The used Chromolith Performance columns	RSD (%) of AUC*	RSD (%) of t_R
pilocarpine hydrochloride	Rod No. 1042/020 Rod No. 1042/012 Rod No. 1043/032 Rod No. 1043/041 Rod No. 1045/036 Rod No. 1045/023	0.30 to 0.94	0.68
propranolol hydrochloride	Rod No. 1042/020 Rod No. 1043/041 Rod No. 1045/036	0.36 to 1.25	0.66
glibenclamide and glimepiride	Rod No. 1042/020 Rod No. 1043/041 Rod No. 1045/036	0.33 to 1.09	0.63
insulin	Rod No. 1042/020 Rod No. 1043/041 Rod No. 5020/028	0.10 to 0.70	0.60

* RSD% range is due to determination at different concentration levels.

3.1.1.2.4 Linearity

The calibration curves (peak area vs. concentration) for the used drugs were investigated over a wide concentration range. Residual plot did not show any trend. Results were found to be linear with high correlation coefficients (Table 12).

Table 12: Linearity of the 5 analyzed compounds.

Compound	Column type	Concentration range (mg/ml)	R^2
pilocarpine hydrochloride	Chromolith Performance RP-18e	0.008 - 0.500	0.9996
	Superspher 100 RP-18e	0.008 - 0.500	0.9998
propranolol hydrochloride	Chromolith Performance RP-18e	0.002 - 1.000	0.9988
	Superspher 100 RP-18e	0.002 - 1.000	0.9992
glibenclamide	Chromolith Performance RP-18e	0.010 - 0.240	0.9998
	Superspher 100 RP-18e	0.010 - 0.240	0.9996
glimepiride	Chromolith Performance RP-18e	0.010 - 0.240	0.9999
	Superspher 100 RP-18e	0.010 - 0.240	0.9999
insulin	Chromolith Performance RP-18e	0.030 - 3.000	0.9998
	Superspher 100 RP-18e	0.030 - 3.000	0.9998

3.1.1.2.5 Detection and quantitation limits

Limit of detection (LOD, S/N = 3) and an estimate for the limit of quantitation (LOQ, S/N = 10) for all the tested drugs on monolithic columns at flow rates of 1 as well as 4 ml/min were found to be lower on the monolithic than on the conventional column. Results of linearity, detection and quantitation limits are summarized in Table 13.

Table 13: Detection and quantitation limits of the 5 analyzed compounds.

Compound	column type	Detection limit(µg/ml)	Quantitation limit (µg/ml)
pilocarpine	Chromolith Performance RP-18e	0.1700	0.500
	Superspher 100 RP-18e	0.3100	1.000
propranolol	Chromolith Performance RP-18e	0.0120	0.040
	Superspher 100 RP-18e	0.0610	0.200
glibenclamide	Chromolith Performance RP-18e	0.1220	0.400
	Superspher 100 RP-18e	0.1240	0.410
glimepiride	Chromolith Performance RP-18e	0.1520	0.506
	Superspher 100 RP-18e	0.1570	0.523
insulin	Chromolith Performance RP-18e	0.0008	0.002
	Superspher 100 RP-18e	0.0013	0.004

3.1.1.3 Performance Parameter

Peak performance parameters for pilocarpine, propranolol, glibenclamide and insulin were also calculated according to fundamental equations, results are shown in Tables 14, 15, 16, and 17, respectively. The least decrease in theoretical plate number (*N*) with increase in flow rate from 1 up to 9 ml/min was found in propranolol method, while the highest decrease was found in glibenclamide method.

Table 14: Performance parameters for pilocarpine on conventional and monolithic columns*.

Column type	Theoretical plate *N* (Plate per column for pilocarpine)	Asymmetry factor for pilocarpine Peak	Resolution (R_s) pilocarpine/pilocarpic acid
Conventional C18 (flow rate 1 ml/min)	1783	1.70	2.10
Monolithic C18 (flow rate 1 ml/min)	3648	1.26	2.78
Monolithic C18 (flow rate 2 ml/min)	3074	1.28	2.33
Monolithic C18 (flow rate 3 ml/min)	2559	1.23	2.23
Monolithic C18 (flow rate 4 ml/min)	2488	1.22	2.13
Monolithic C18 (flow rate 5 ml/min)	2415	1.21	1.92
Monolithic C18 (flow rate 6 ml/min)	1950	1.35	1.80
Monolithic C18 (flow rate 7 ml/min)	1777	1.40	1.58
Monolithic C18 (flow rate 8 ml/min)	1430	1.32	1.43
Monolithic C18 (flow rate 9 ml/min)	1336	1.30	1.32

*The following equations were used to calculate the above mentioned chromatographic parameters: (N = 16 $(t_R/w)^2$, asymmetry factor (AF) = A/B at 10% of peak height (A & B are the two half widths of the peak center at each side at 10% height from the peak base) and resolution $R_s = 2(t_{R2}-t_{R1}/w_2+w_1)$.

Table 15: Performance parameters for propranolol on conventional and monolithic columns*.

Column type	Theoretical plate *N* (Plate per column for propranolol hydrochloride)	Asymmetry factor for propranolol Peak	Resolution (R_s) propranolol impurity A/propranolol
Conventional C18 (flow rate 1 ml/min)	379	1.4	4.39
Monolithic C18 (flow rate 1 ml/min)	1120	1.1	4.40
Monolithic C18 (flow rate 2 ml/min)	980	1.1	4.18
Monolithic C18 (flow rate 3 ml/min)	925	1.1	3.26
Monolithic C18 (flow rate 4 ml/min)	867	1.2	2.80
Monolithic C18 (flow rate 5 ml/min)	858	1.1	2.00
Monolithic C18 (flow rate 6 ml/min)	792	1.1	1.94
Monolithic C18 (flow rate 7 ml/min)	769	1.1	1.80
Monolithic C18 (flow rate 8 ml/min)	757	1.2	1.66
Monolithic C18 (flow rate 9 ml/min)	694	1.1	1.58

*The following equations were used to calculate the above mentioned chromatographic parameters: ($N = 16\ (t_R/w)^2$, asymmetry factor (AF) = A/B at 10% of peak height (A & B are the two half widths at each side of the peak center) and resolution $R_s = 2(t_{R2}\text{-}t_{R1}/w_2+w_1)$

Compared to the traditional particulate column, monolithic columns were found to produce the same or better resolution and peak symmetry in a shorter run time (taking in account the small difference in column length) at the same flow rate of 1 ml/min. With the application of higher flow rates on Chromolith Performance columns small reductions in resolution were observed. However at a flow rate of 4 ml/min the resolution obtained with the monolithic columns was still convenient ($R_s \geq 2$) with the advantage of strongly reducing the total runs time. This reduces the time consumption for routine series of analyses. Representative chromatograms for the four drug methods (pilocarpine, propranolol, glibenclamide-glimepiride and insulin) on

conventional and monolithic columns are shown in Figures 23, 24, 25 and 26, respectively. The peak symmetry was better in case of monolithic columns compared to the conventional column. The height equivalent to theoretical plate was used to measure efficiency depending on flow rate.

Table 16: Performance parameters for glibenclamide and the two related compounds on conventional and monolithic columns*.

Column type	Theoretical plate *N* (Plate per column for glibenclamide)	Asymmetry factor for glibenclamide Peak	Resolution (R_s) Compound a /compound b
Conventional C18 (flow rate 1,25 ml/min)	4435	1.8	3.40
Monolithic C18 (flow rate 1,25 ml/min)	7964	1.0	4.10
Monolithic C18 (flow rate 2ml/min)	7540	1.1	3.86
Monolithic C18 (flow rate 3 ml/min)	6725	1.1	3.20
Monolithic C18 (flow rate 4 ml/min)	6151	1.0	2.80
Monolithic C18 (flow rate 5 ml/min)	5807	1.0	2.40
Monolithic C18 (flow rate 6 ml/min)	5003	1.1	1.90
Monolithic C18 (flow rate 7 ml/min)	4328	1.0	1.70
Monolithic C18 (flow rate 8 ml/min)	3755	1.0	1.40
Monolithic C18 (flow rate 9 ml/min)	2822	1.1	1.13

*The following equations were used to calculate the above mentioned chromatographic parameters: (N =16 $(t_R/w)^2$, asymmetry factor (AF) = A/B at 10% of peak height (A & B are the two half widths at each side of the peak center) and resolution $R_s = 2(t_{R2}-t_{R1}/w_2+w_1)$.

Table 17: Performance parameters for human insulin on conventional and monolithic columns.*

Column type	Theoretical plate *N* (Plate per column for human insulin)	Asymmetry factor for human insulin Peak	Resolution (R_s) human insulin/21-desamido insulin
Conventional C18 (flow rate 1 ml/min)	1665	1.2	2.60
Monolithic C18 (flow rate 1 ml/min)	3003	1.2	4.50
Monolithic C18 (flow rate 2ml/min)	2809	1.2	4.10
Monolithic C18 (flow rate 3 ml/min)	2605	1.2	3.95
Monolithic C18 (flow rate 4 ml/min)	2379	1.1	3.74
Monolithic C18 (flow rate 5 ml/min)	2174	1.0	3.49
Monolithic C18 (flow rate 6 ml/min)	2077	1.0	3.35
Monolithic C18 (flow rate 7 ml/min)	1808	1.1	3.32
Monolithic C18 (flow rate 8 ml/min)	1615	1.1	3.27
Monolithic C18 (flow rate 9 ml/min)	1478	1.0	3.20

*The following equations were used to calculate the above mentioned chromatographic parameters: (N =16 $(t_R/w)^2$, asymmetry factor (AF) = A/B at 10% of peak height (A & B are the two half widths at each side of the peak center) and resolution $R_s = 2(t_{R2}-t_{R1}/w_2+w_1)$

Column efficiency was measured by plotting the height equivalent to theoretical plates which is abbreviated as (HETP) or simply referred to as plate height (H) against the flow rates of the mobile phase (Figure 27). The plate height H was calculated from the column length L and theoretical plate N according to $H = L/N$.

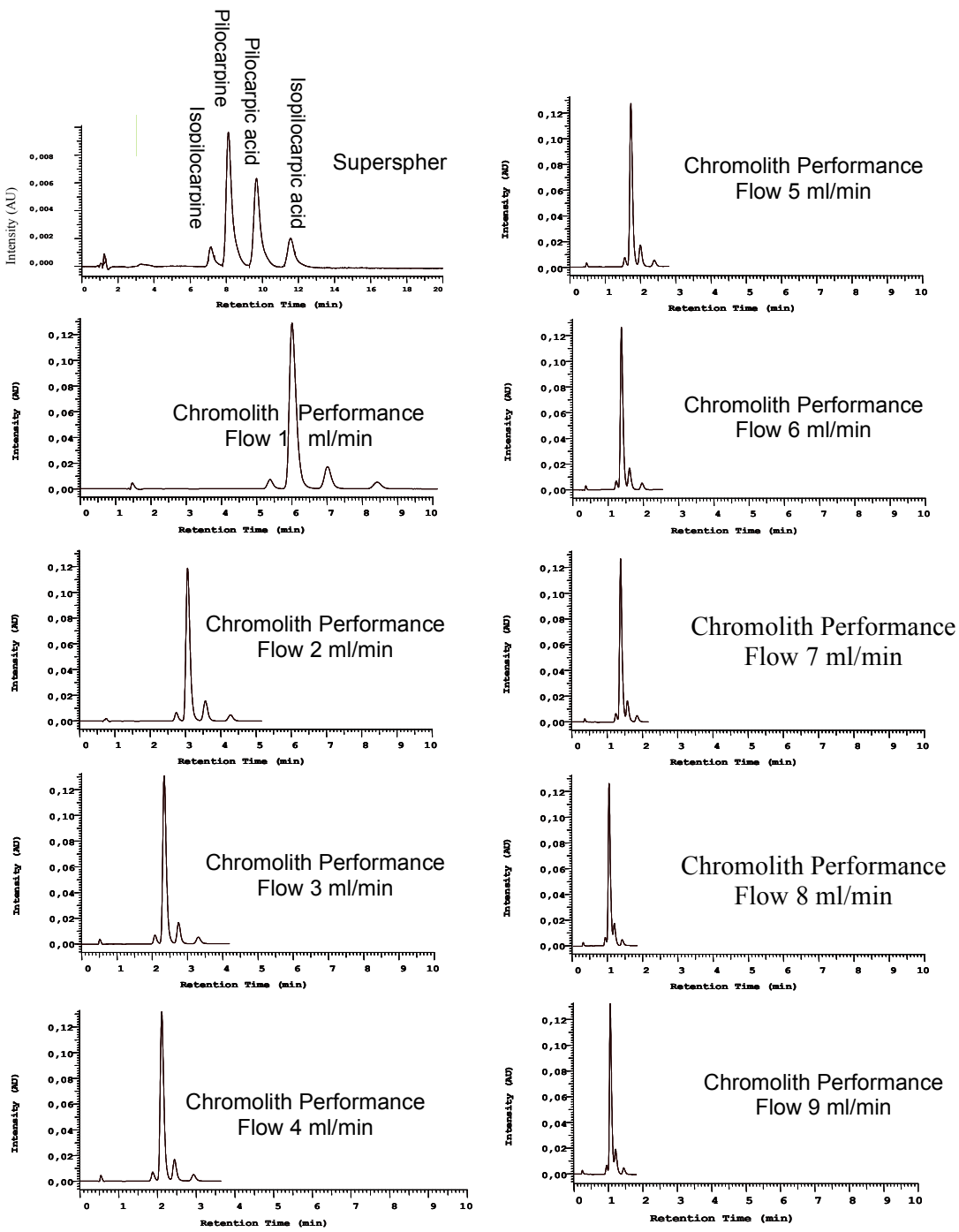


Figure 23: Representative chromatograms for pilocarpine hydrochloride and its degradation products on conventional (Superspher RP-18e) column, and on monolithic (Chromolith Performance RP-18e) column at different flow rates from 1 to 9 ml/min. Mobile phase consists of a buffer pH = 3: methanol (980:20, v/v). Difference in peak intensity between conventional and monolithic columns is due to difference in concentration of pilocarpine and its degradation products.

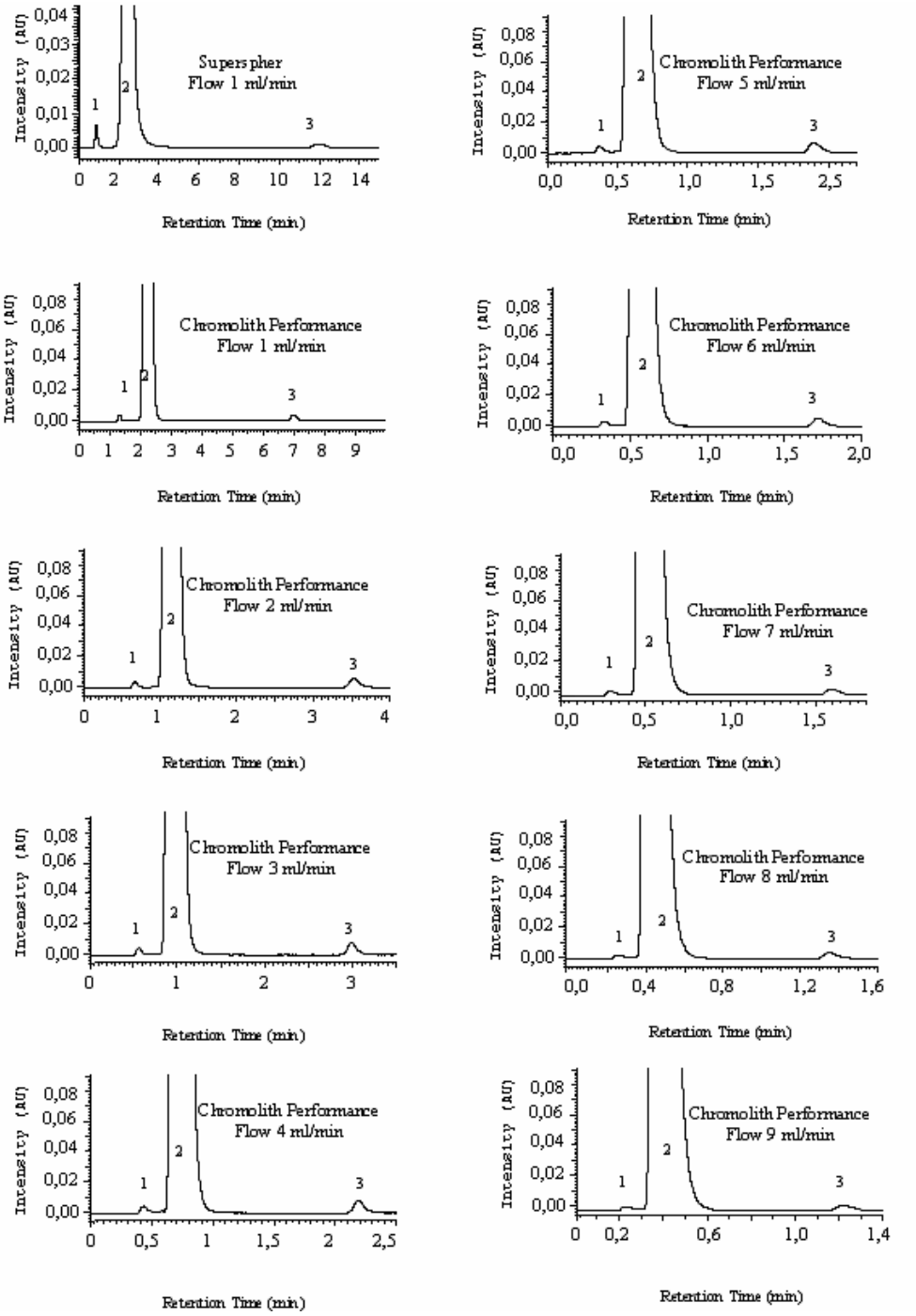


Figure 24: Representative chromatograms for propranolol hydrochloride (peak 2) and its two Degradation products a & b (peaks 1 & 3, respectively) on conventional (Superspher RP-18e) and on Monolithic (Chromolith Performance RP-18e). Mobile phase consists of buffer pH = 3.3: acetonitrile (40:60, v/v).

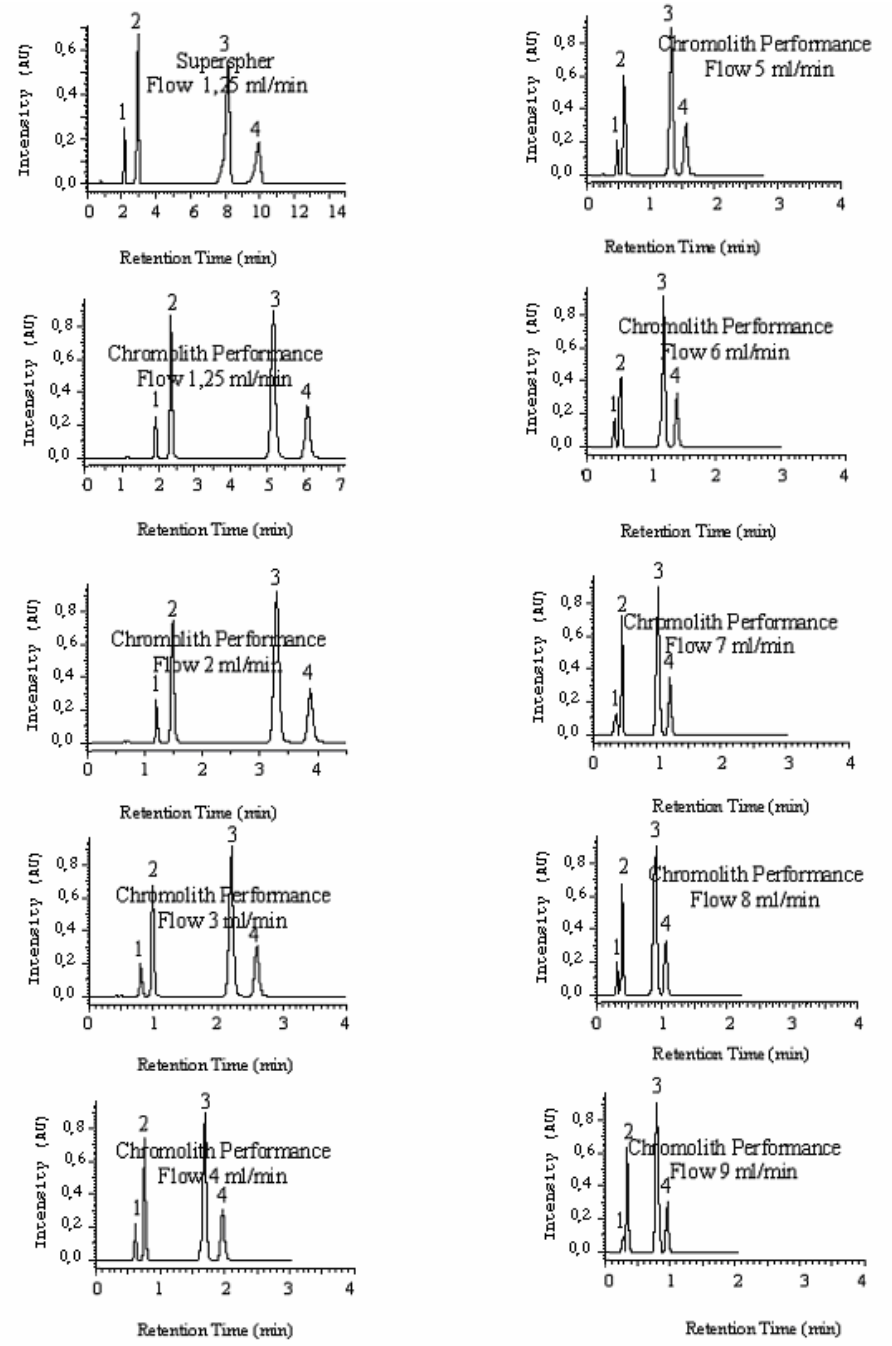


Figure 25: Representative chromatograms for related compound a (1) related compound b (2), glibenclamide (3) & glimepiride (4) on conventional (Superspher RP-18e) and on monolithic (Chromolith Performance RP-18e) columns at different flow rates from 1,25 to 9 ml/min. Mobile phase consist of buffer pH = 3:acetonitrile (55:45, v/v).

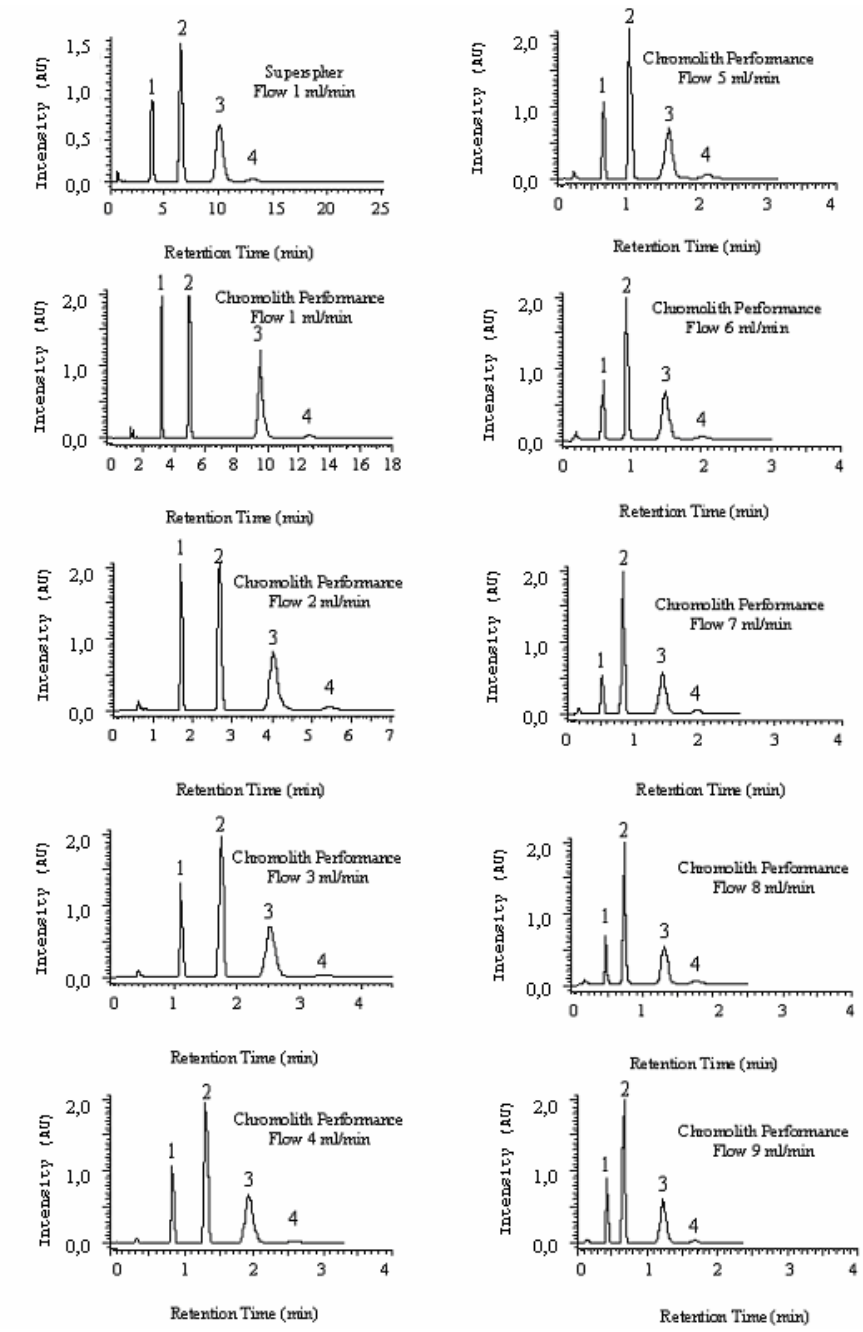


Figure 26: Representative chromatograms for phenol (1), m-cresol (2), human insulin (3) & 21-desamido human insulin (4) on conventional (Superspher RP-18e) and on monolithic (Chromolith Performance RP-18e) columns at different flow rates from 1 to 9 ml/min. Mobile phase consist of buffer pH = 2.3: acetonitrile in a ratio of (74:26, v/v) and (74.5:25.5, v/v) for conventional and monolithic columns, respectively.

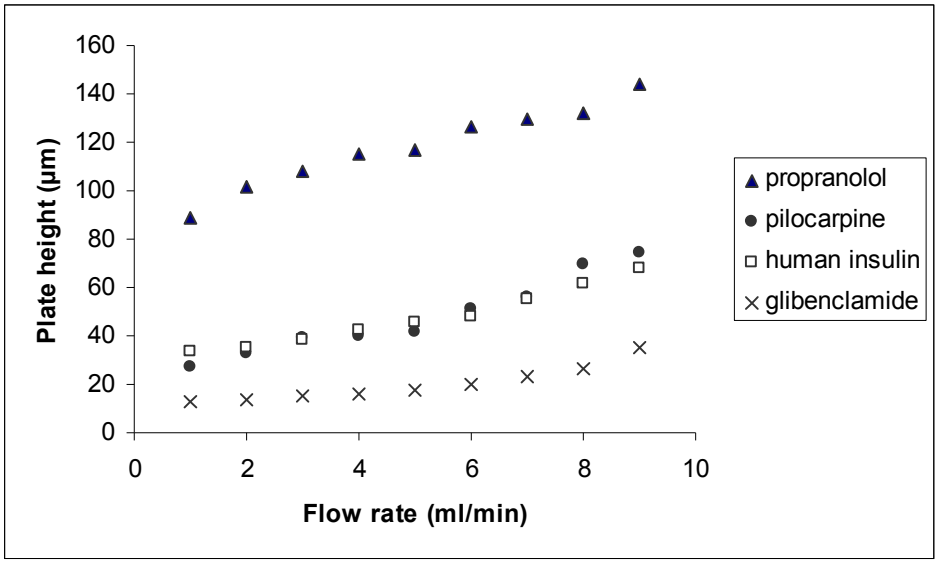


Figure 27: Plot of the height equivalent to theoretical plate against mobile phase flow rate, for pilocarpine, propranolol, glibenclamide and insulin on a Chromolith Performance RP-18e column.

3.1.2 Results of high speed analysis with flow programming

3.1.2.1 Optimization of chromatographic parameters

3.1.2.1.1 Flow rate

Looking back to the chromatograms of glibenclamide and related products shown in Figure 25, the 4 compounds were eluted within 66 seconds at a flow rate of 9 ml/min. However, there was a loss of resolution between the peaks of related compounds a and b (peaks 1 and 2). In an aim to improve the resolution between compound a and compound b at a flow rate of 9 ml/min, the elution strength of the mobile phase was modified by decreasing the acetonitrile percentage from 45% to 42%. The resolution was improved but the chromatographic run time was increased to about 2.2 min. Thus the acetonitrile content was kept at 45%. In order to obtain a complete separation between the 4 compounds at the shortest analysis time, a flow program was used ranging from 5.0 to 9.9 ml/min to allow a rapid elution of the 4 compounds without scarifying resolution. A stepwise increase of the flow rate from 5 to 6 ml/min was applied at the first 35 seconds to avoid the loss of resolution between the closely related compounds a and b. After that, the flow rate was increased gradually to 9.9 ml/min to accelerate the elution of the late eluting glibenclamide and glimepiride (Table 18).

Table 18: Flow rate program used during the separation

Time (min)	Flow rate (ml/min)
0.0	5.0
0.6	6.0
0.7	9.0
1.3	9.9

The separation was accomplished within 80 seconds. The 4 compounds were well separated from each other with a resolution value R_s = 2.2 between the critical peak pairs a and b. A representative chromatogram for the fast separation of glibenclamide, glimepiride and their two related compounds is shown in Figure 28.

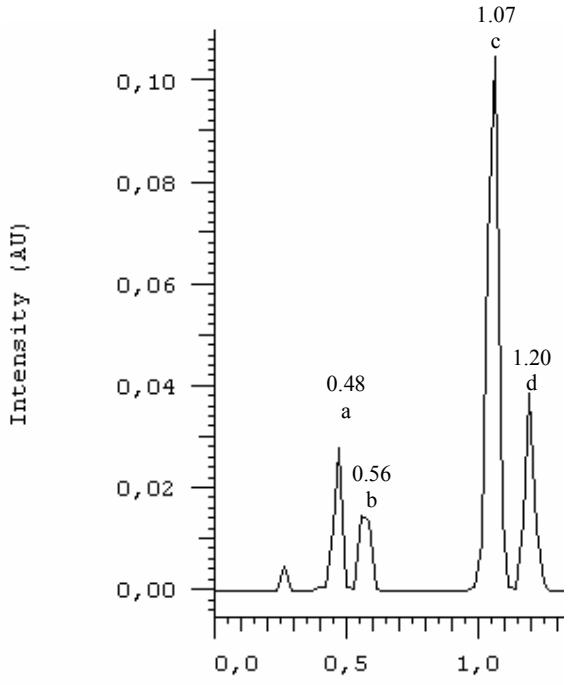


Figure 28: Representative chromatograms for related compound a (a) related compound b (b), glibenclamide (c) and glimepiride (d) on a Chromolith Performance RP-18e column at a flow rate program (5.0 - 9.9 ml/min) and a column temperature of 30°C. Mobile phase consist of phosphate buffer pH = 3: acetonitrile (55:45, v/v).

3.1.2.1.2 Temperature effect

The temperature of the column oven was set to 30°C because no further improvement in peak broadening or decrease in retention times was observed at higher temperatures, even when a mobile phase water bath was used in combination with the column oven. Temperatures were tested up to 45°C using this flow rate program. The effect of temperature was only minor but measurable at the low flow rate of 1.25 ml/min at which the retention times for all the 4 peaks was reduced using higher temperature. Representative chromatograms of the analyzed mixture at a flow rate of 1.25 ml/min using column oven set to 30 °C and to 45 °C are shown in Figure 29. No change in the resolution or precision was observed with a slight variation in buffer pH in the range of 2.5 – 3.5.

3.1.2.2 Method validation

3.1.2.2.1 Specificity

The specificity of the method was examined by observing if there was any interference from the inactive ingredients. The HPLC chromatograms recorded for the inactive ingredients showed no peaks at the retention times of the 4 compounds.

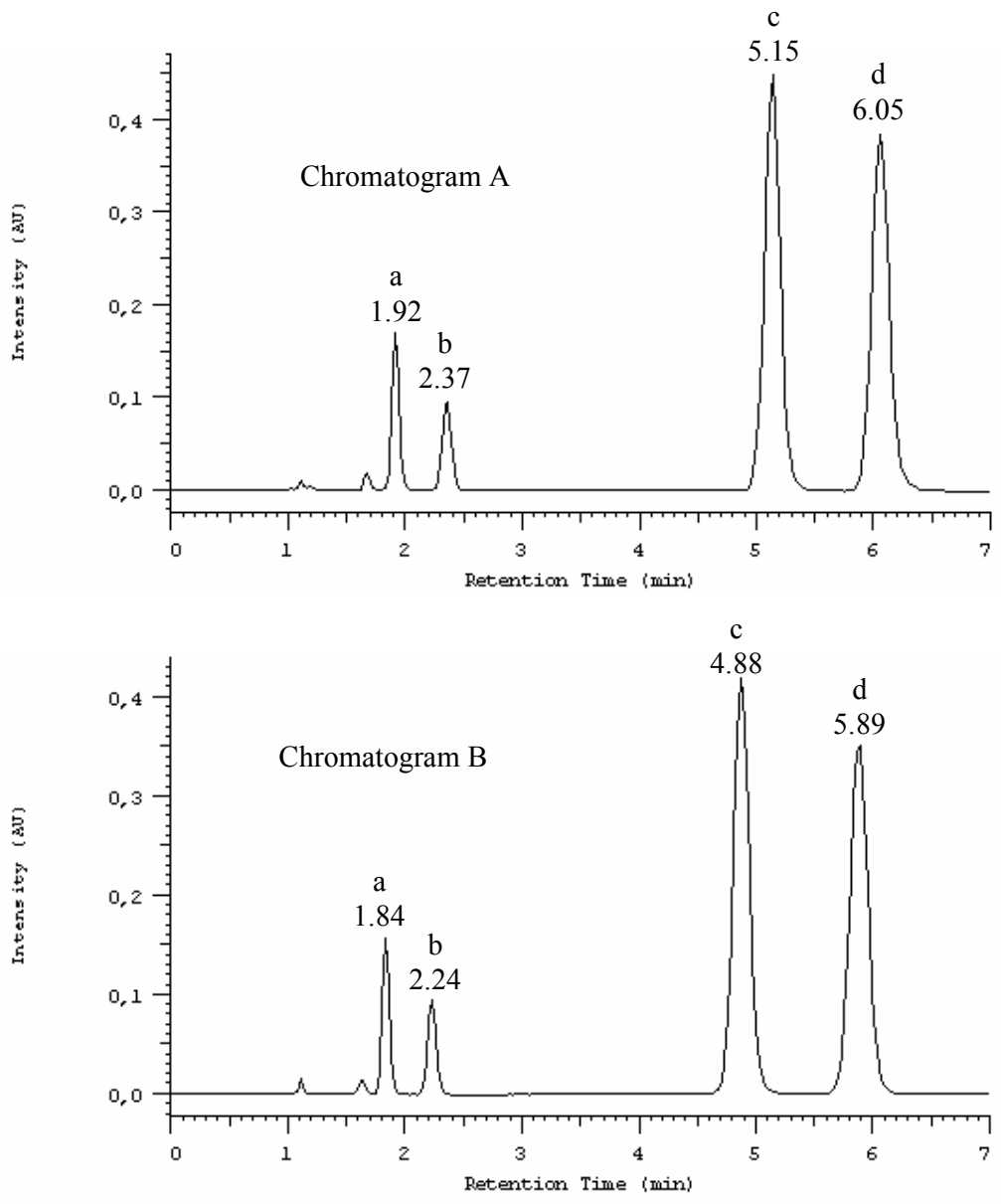


Figure 29: Representative chromatograms for related compound a (a) related compound b (b), glibenclamide (c) and glimepiride (d) on a Chromolith Performance RP-18e column at a flow rate 1.25 ml/min and a column temperature of 30°C for (Chromatogram A), and 45°C for (Chromatogram B). Mobile phase consist of buffer pH = 3: acetonitrile (55:45, v/v).

3.1.2.2.2 Accuracy

The accuracy of the method was tested by determination of the recovery using the inactive ingredient used in glibenclamide and glimepiride tablet formulations. Good recovery percentages were obtained for both glibenclamide and glimepiride. The recovery results for both glibenclamide and glimepiride at three concentration levels are summarized in Table 19.

Table 19: List of recovery results (n = 10) for glibenclamide and glimepiride from synthetic mixture of drug product compounds at three concentration levels.

Compound	Theoretical value (mg/ml)	Mean recovery (mg/ml)	Recovery %	RSD %
glibenclamide	0.16	0.1590	99.34	0.85
	0.20	0.1990	99.95	0.56
	0.24	0.2409	100.4	0.79
glimepiride	0.16	0.1603	100.2	0.92
	0.20	0.1988	99.89	0.78
	0.24	0.2370	99.10	1.00

3.1.2.2.3 Precision

To ensure assay precision within day repeatability (n = 5) and between days repeatability (n = 5) were assessed at 3 concentration levels for each of the four compounds. The RSDs % was found to be < 1.0% for retention time and < 1.2% for peak area (Table 20).

3.1.2.2.4 Linearity

Calibration curves (peak area vs. concentration) for the four analyzed compounds in sample solvent were investigated over a wide concentration range. Results are summarized in Table 21.

3.1.2.2.5 Detection and quantitation limits

The limit of detection (LOD, S/N = 3) and an estimate for the limit of quantitation (LOQ, S/N=10) for the 4 analyzed compounds were also summarized in Table 21.

Table 20: Precision over a concentration range 0.001 - 0.100 mg/ml for related compound a and b and 0.01 - 0.24 mg/ml for glibenclamide and glimepiride using n=5 for both within day and between days repeatabilities.

Compound	Within day repeatability RSD range of AUC	Within day repeatability RSD of t_R	Between day repeatability RSD range of AUC	Between day repeatability RSD of t_R
related compound a	0.40% - 0.62%	0.37%	0.58% - 0.79%	0.93%
related compound b	0.50% - 0.62%	0.80%	0.68% - 1.14%	0.98%
glibenclamide	0.45% - 0.60%	0.41%	0.76% - 0.85%	0.58%
glimepiride	0.36% - 0.56%	0.58%	0.63% - 1.03%	0.94%

Table 21: Linearity, detection and quantitation limits of the 4 analyzed compounds.

Compound	Concentration range (mg/ml)	R^2	Detection limit (µg/ml)	Quantitation limit (µg/ml)
related compound a	0.0002 - 0.1	0.9981	0.048	0.16
related compound b	0.0002 - 0.1	0.9998	0.050	0.18
glibenclamide	0.001 - 0.24	0.9998	0.120	0.41
glimepiride	0.001 - 0.24	0.9981	0.150	0.50

3.1.2.3 Performance parameter

Peak performance parameters were calculated according to fundamental equations (Table 22). The following equations were used to calculate the mentioned chromatographic parameters mentioned in Table 22: (N = 16 $(t_R/w)^2$, asymmetry factor (AF) = A/B at 10% of peak height and Resolution R_s = 2 $(t_{R2}\text{-}t_{R1}/w_2\text{+}w_1)$. A slow decrease in resolution was observed by applying high flow rates using monolithic column. Column efficiency was measured by plotting the plate height (H) against the flow rates of the mobile phase. The height equivalent to theoretical plate H was calculated from the column length L and theoretical plate N according to $H = L/N$.

Table 22: Performance parameters for glibenclamide and the two related compounds on conventional and monolithic columns.*

Column type	Theoretical plate *N* (plate per column for glibenclamide)	Asymmetry factor for glibenclamide peak	Resolution (R_s) compound a/compound b	Total run time (min)
Conventional C18 (flow rate 1.25 ml/min)	4435	1.8	3.40	11.0
Monolithic C18 (flow rate 1.25 ml/min)	7964	1.0	4.10	6.5
Monolithic C18 (flow rate 2 ml/min)	7540	1.1	3.86	4.5
Monolithic C18 (flow rate 3 ml/min)	6725	1.1	3.20	3.0
Monolithic C18 (flow rate 4 ml/min)	6151	1.0	2.80	2.5
Monolithic C18 (flow rate 5 ml/min)	5807	1.0	2.40	1.8
Monolithic C18 (flow rate 6 ml/min)	5003	1.1	1.90	1.6
Monolithic C18 (flow rate 7 ml/min)	4328	1.0	1.70	1.4
Monolithic C18 (flow rate 8 ml/min)	3755	1.0	1.40	1.3
Monolithic C18 (flow rate 9 ml/min)	2822	1.1	1.13	1.2
Monolithic C18 (flow program 5.0 – 9.9 ml/min)	5288	1.0	2.20	1.3

Flat curves for plate height versus linear velocity curves were obtained for the four analyzed compounds (Figure 30). This indicates that monolithic columns can operate at high flow rates with only small decrease in efficiency. The high permeability of the monolithic columns was evidenced by a total system back pressure of 253 bar at a flow rate of 9.9 ml/min. This high flow rate is not applicable in case of conventional particle-packed columns. The backpressure profile during the flow program of 5 to 9.9 ml/min is shown in Figure 31. This backpressure profile during the flow program of 5 to 9.9 ml/min ranged from 144 to 250 bar.

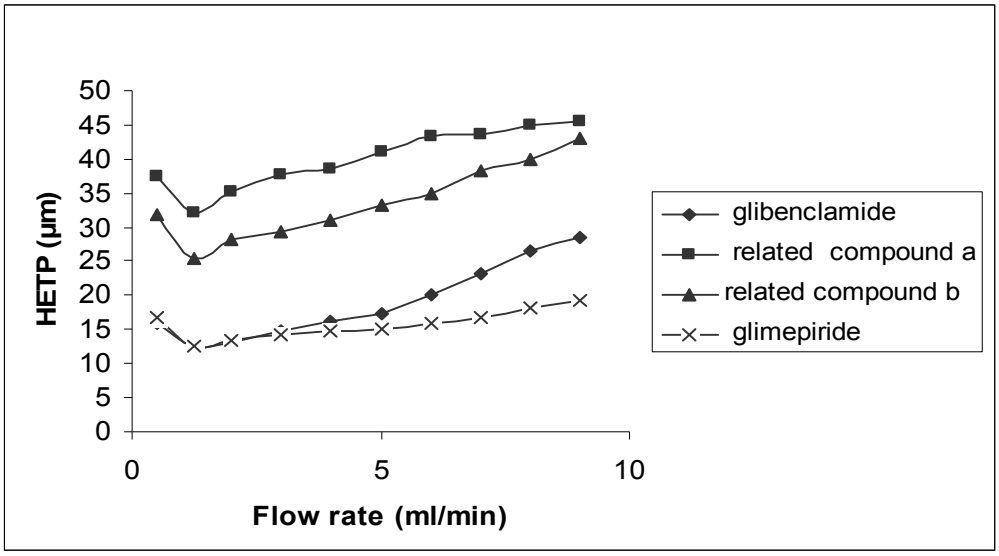


Figure 30: Van Deemter plot for the 4 analyzed compounds on Chromolith Performance column.

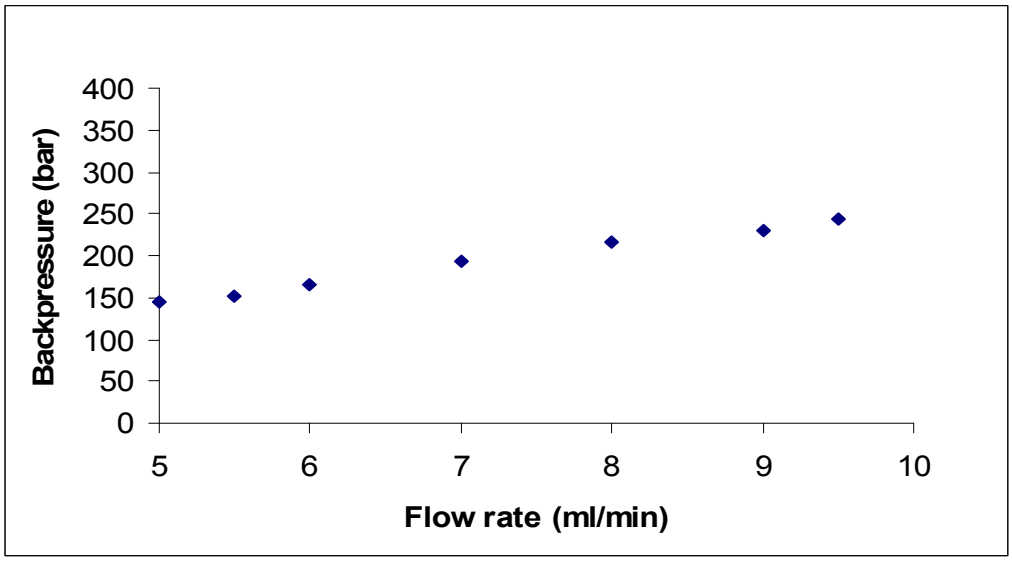


Figure 31: Backpressure during the flow rate program (5.0 - 9.9 ml/min). Mobile phase consist of phosphate buffer pH = 3: acetonitrile (55:45, v/v).

A comparison between backpressure on conventional and monolithic columns is shown in Figure 32. In conventional columns, the maximum acceptable backpressure of 400 bar is reached at a flow rate of about 4 ml/min depending on the column length and the mobile phase composition.

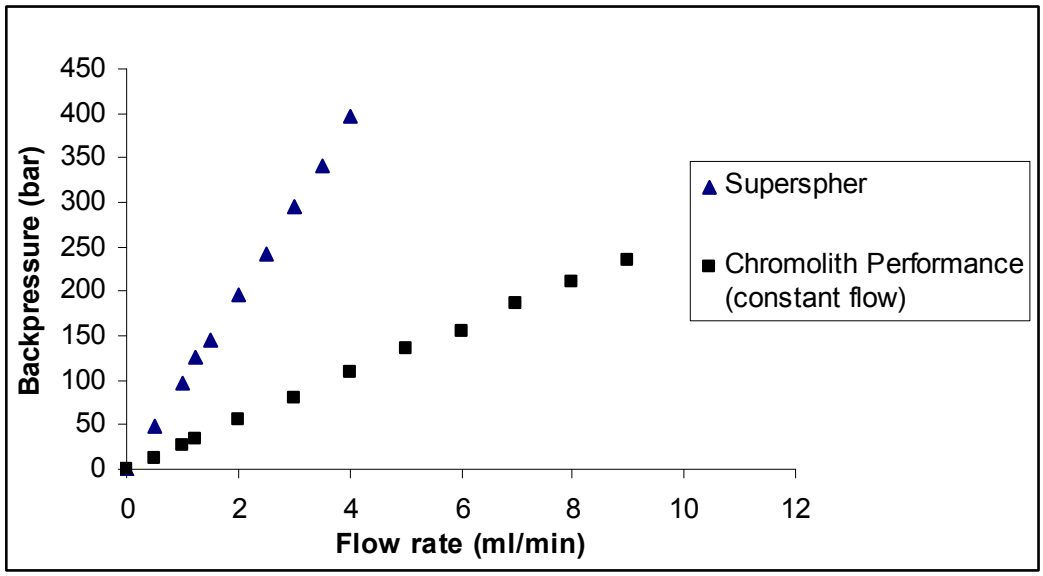


Figure 32: Plot of column backpressure against flow rate of mobile phase for conventional particle-packed (Superspher RP-18e) and monolithic (Chromolith Performance RP-18e) columns. Mobile phase consist of phosphate buffer pH = 3: acetonitrile (55:45, v/v).

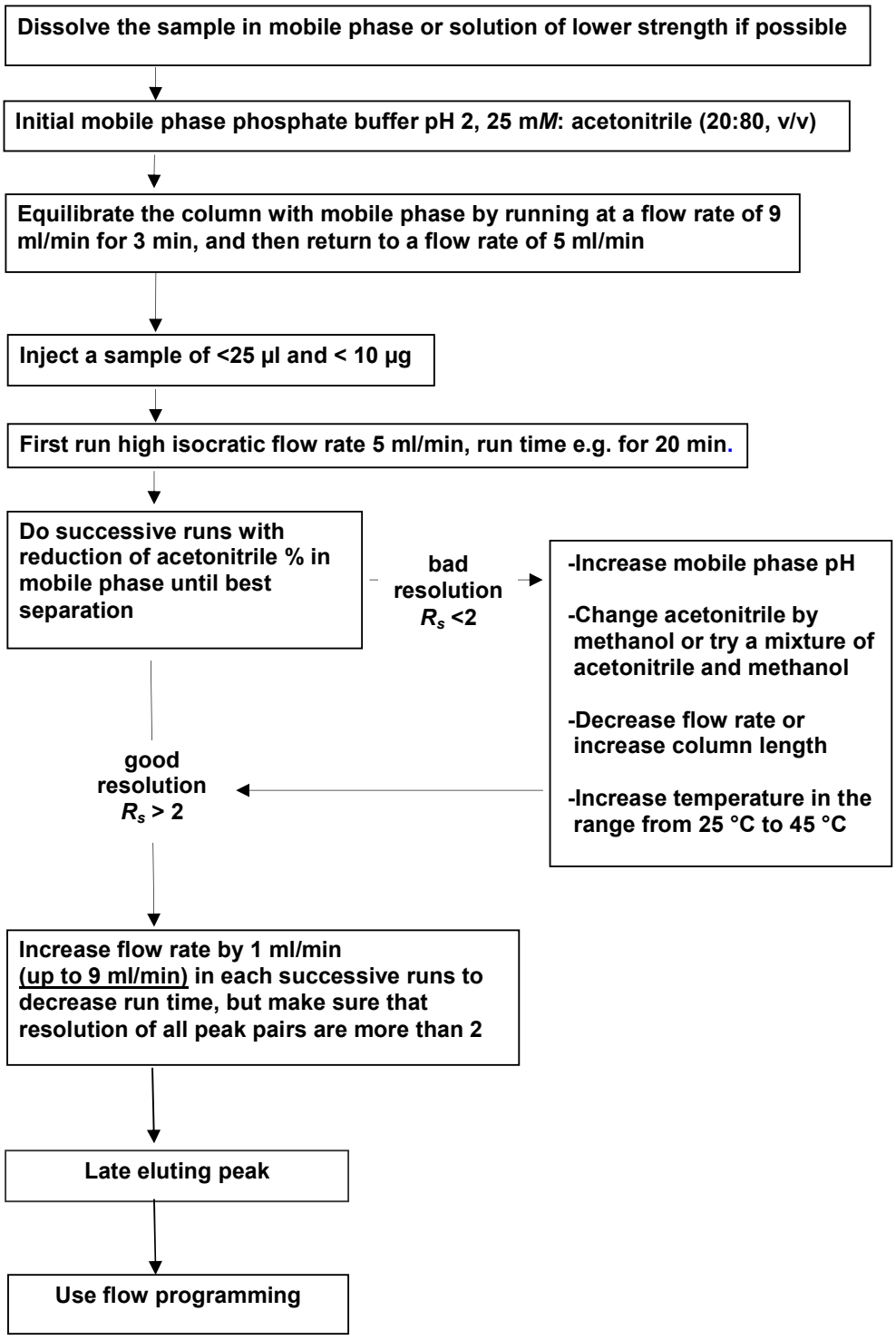
3.1.3 Result method development with monolithic columns

3.1.3.1 Examples for developed methods

Heir a general strategy is derived for developing a method using monolithic column when no previous method on a conventional column is available. The strategy will of course depend on the use of high flow rates and the possibility of using flow programming offered by monolithic silica columns.

The steps for RP-HPLC method development using monolithic silica columns have been summarized in Chart 2. Examples outlined in this section show how monolithic columns can provide fast RP-HPLC method development which would not be possible with conventional columns.

Chart 2: Strategy for method development using monolithic silica columns.



3.1.3.1.1 Separation of acidic mixture

The first example shows the separation of seven acidic compounds using monolithic silica columns. An initial flow rate of 5 ml/min was used with a mobile phase

consisting of acetonitrile: phosphate buffer pH 2 (80:20). The chromatogram obtained under this condition showed only one peak for the whole mixture indicating a co-elution of the compounds altogether (Figure 33 a). Successive runs were tried with a 60%, 40%, 20%, 10% and 5% acetonitrile in the mobile phase.

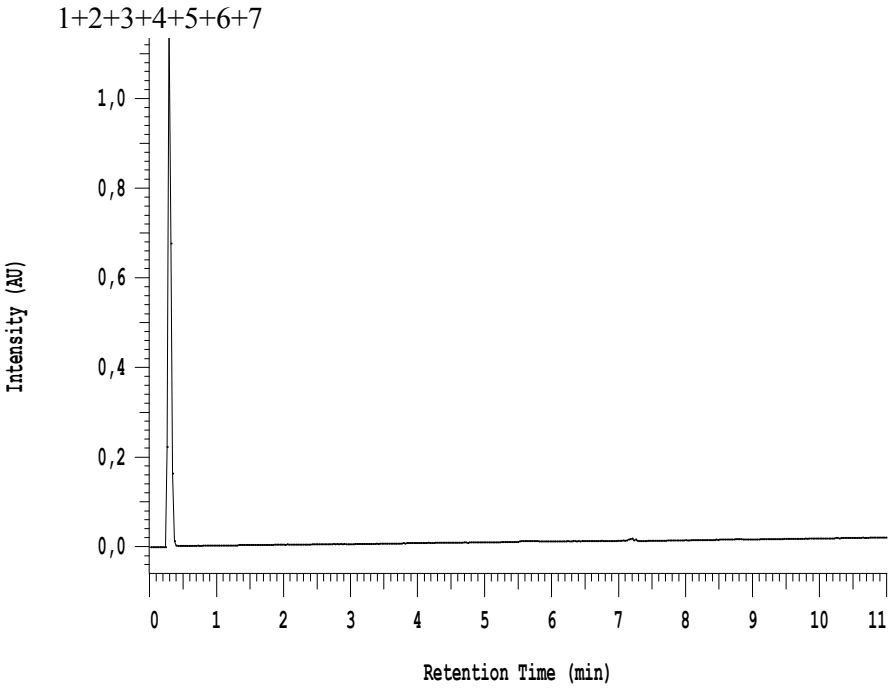


Figure 33 a: Separation of seven acidic compounds. Column: Chromolith Performance, eluent: Acetonitrile-25m*M* phosphate buffer pH 2 (80: 20). Flow rate: 5ml/min and detection wavelength 273 nm. Nicotinic acid (1), resorcin (2), phenol(3), salicylic acid (4), benzoic acid(5), 4-hydroxy-benzoic acid (6) and 2-Naphthol (7).

The best separation was obtained by 10% acetonitrile but only 5 peaks appeared for the 7 compounds because nicotinic acid, resorcin, and phenol co-eluted together (Figure 33 b). No further improvement was obtained by decreasing the acetonitrile content to 5%. On the other hand, no better separation was obtained by increasing the pH to 3, 4, or 5 using acetonitrile as organic modifier. After replacing the best obtained ratio of acetonitrile (10%) by methanol (methanol:phosphate pH 2, 10:90), also only six peaks appear because the peaks for salicylic and benzoic acids overlapped (Figure 33 c).

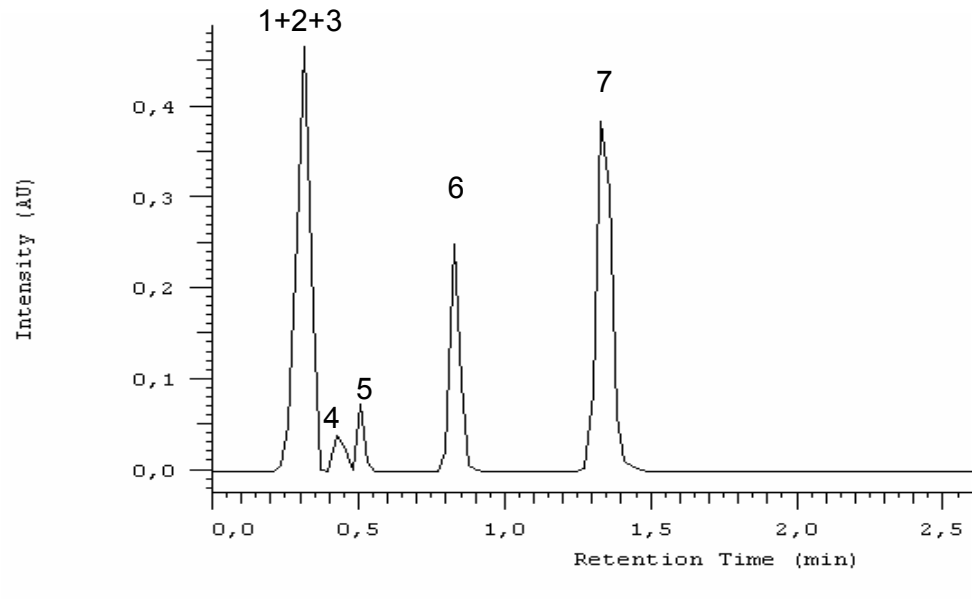


Figure 33 b: Separation of seven acidic compounds. Column: Chromolith Performance, eluent: acetonitrile-25m*M* phosphate buffer pH 2 (10: 90). Flow rate: 5ml/min and detection wavelength 273 nm. Nicotinic acid (1), resorcin (2), phenol(3), salicylic acid (4), benzoic acid(5), 4-hydroxy benzoic acid (6) and 2-naphthol (7).

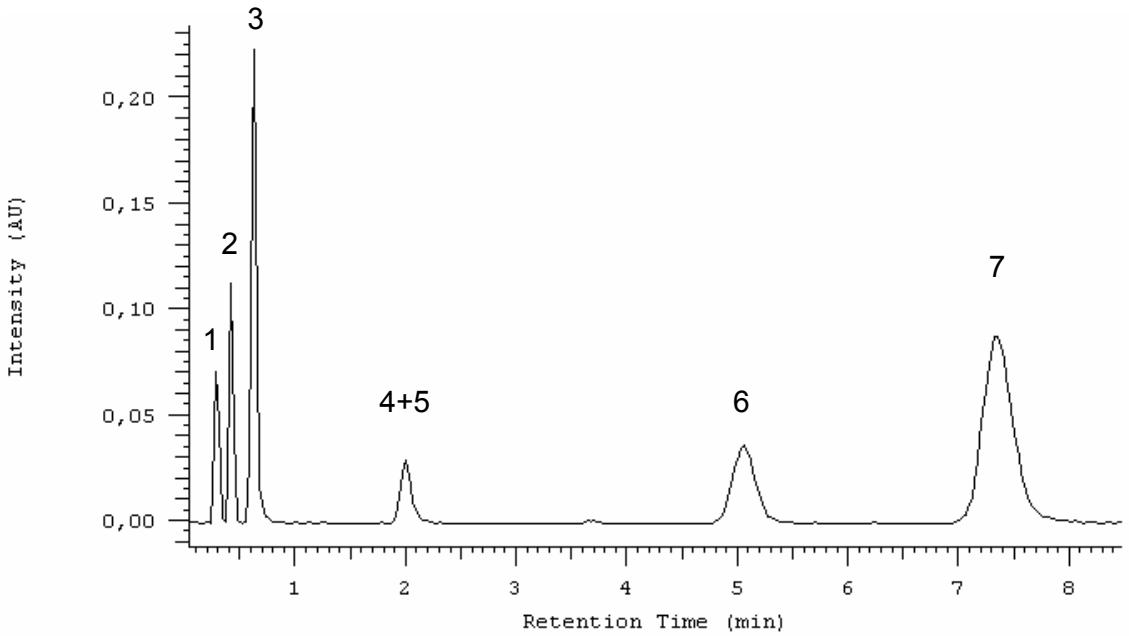


Figure 33 c: Separation of seven acidic compounds. Column: Chromolith Performance, eluent: methanol-25m*M* phosphate buffer pH 2 (10: 90). Elution order as in Figure 33 b.

The best separation was obtained using pH 3 and 10% methanol in the mobile phase (Figure 33 d). Further reduction of the run time was achieved using an appropriate flow program of Table 23 as shown in chromatogram of Figure 33 e.

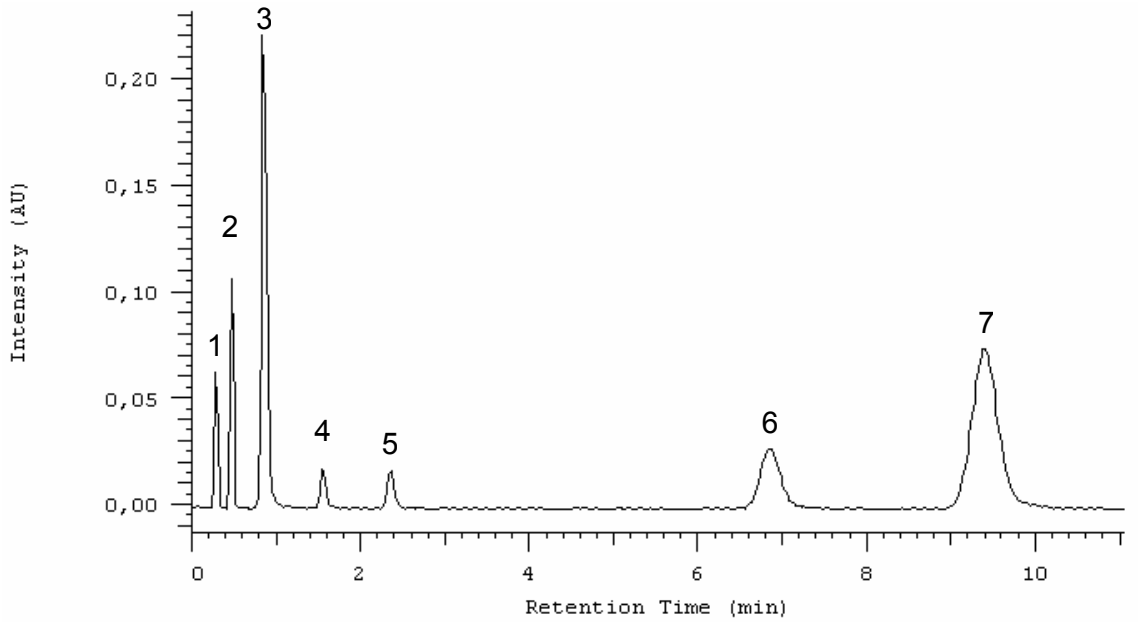


Figure 33 d: Separation of seven acidic compounds. Column: Chromolith Performance, eluent: methanol:25 m*M* phosphate buffer pH 3 (10: 90). Flow rate: 5ml/min and detection wavelength 273 nm. Peak names as Figure 33 b.

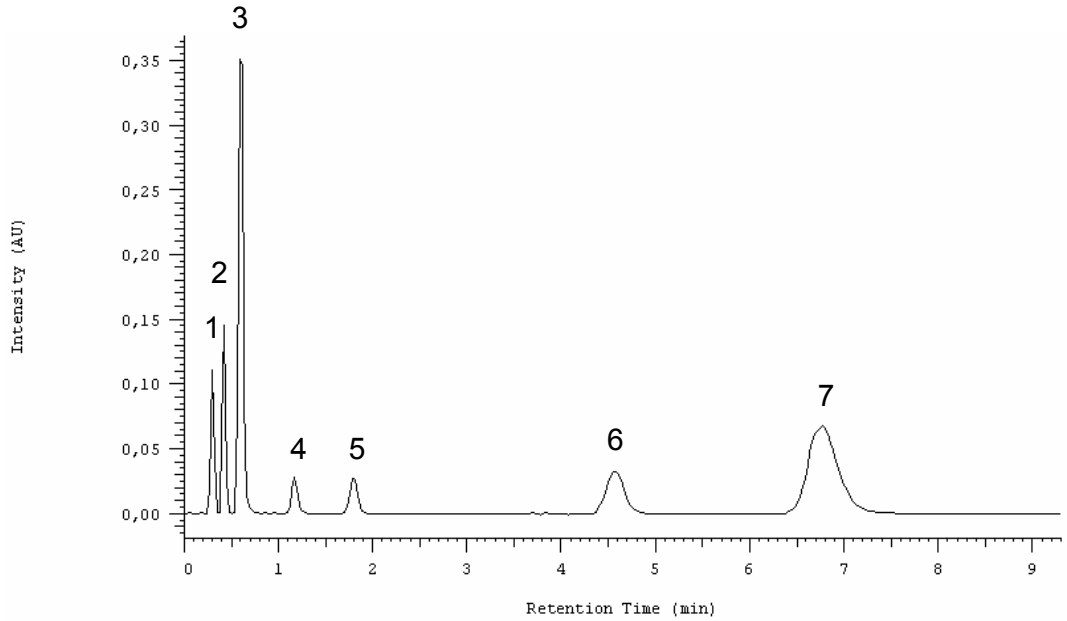


Figure 33 e: Separation of a seven acidic compounds. Column: Chromolith Performance, eluent: methanol-25m*M* phosphate buffer pH 3 (10: 90) using the flow program shown in the Table 23. Peak names as Figure 33 b.

Table 23: Flow program used for separation of acidic mixture in Figure 33 e.

Time (min)	Flow rate (ml/min)
0.0	5
1.0	6
1.1	9
8.0	9

3.1.3.1.2 Separation of aniline and five derivatives

Figure 34 shows the separation of a basic mixture of aniline and five derivatives. As a starting condition a mobile phase of methanol: phosphate buffer pH 2 (80:20) was used. Best resolution was obtained after reducing methanol to 45% and increasing the pH to 5. Under this condition the 6 compounds were separated within 4.5 min using the flow program mentioned in Table 24.

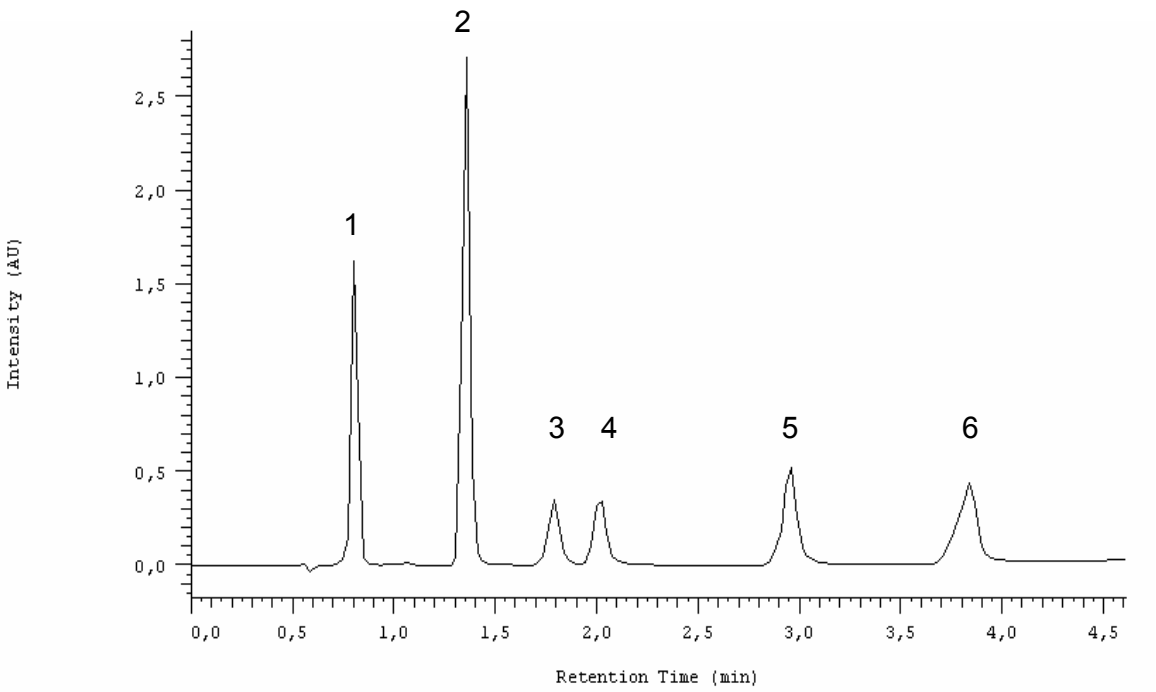


Figure 34: Separation of six basic compounds aniline and five derivatives. Column:Chromolith Performance RP-18e, eluent: phosphate buffer pH 5, 25 m*M*: methanol (55:45), detection 214 nm, using the flow program shown in Table 24. (1) Aniline (2) N-methylaniline (3) N-ethylaniline (4) 4-ethylaniline (5) dimethylaniline (6) N,N-diethylaniline.

Table 24: Flow program used for separation of aniline and its derivatives in Figure 34.

Time (min)	Flow rate (ml/min)
0,0	3.0
2.1	3.0
2.2	9.0
5.0	9.0

3.1.3.1.3 Separation of alkaloid mixture

Figure 35 a shows the separation of a challenging basic alkaloid mixture. This chromatogram was the best one obtained after trying several pH values from pH 2 to pH 8 in the mobile phase. However, peaks 1, 2 and 3 are still unresolved. Resolution of this basic mixture seems to require a high basic pH value in the mobile phase which interferes with the column stability.

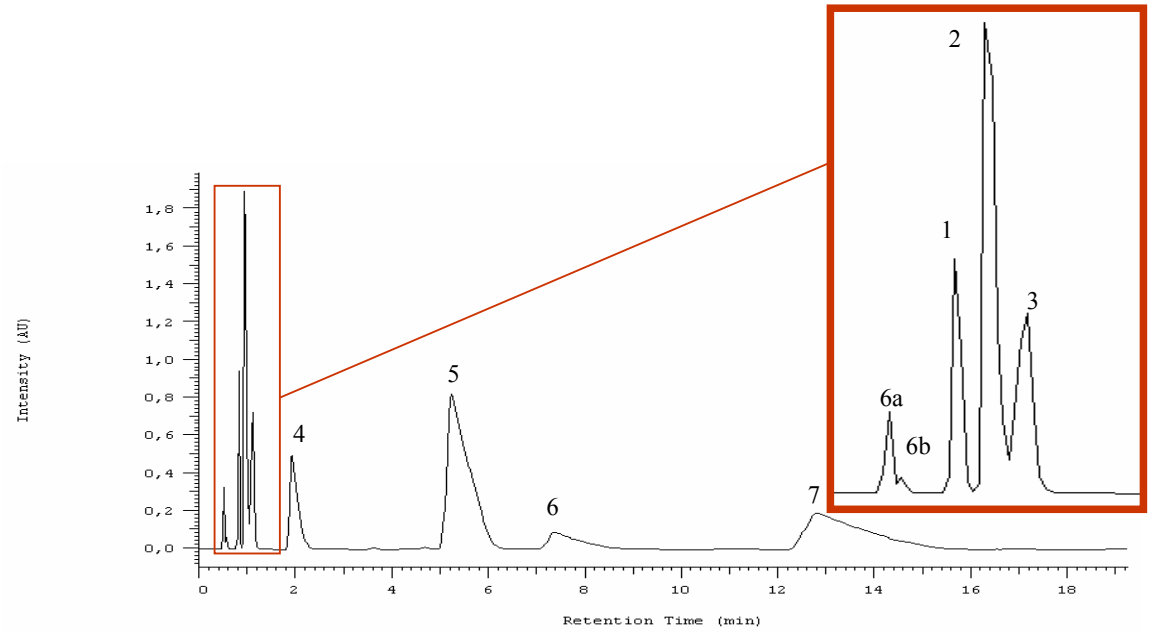


Figure 35 a: Separation of a basic mixture of seven alkaloids. Column: Chromolith Performance Rp-18e, eluent: phosphate buffer 25m*M*, pH 3: methanol (80:20), flow rate: 3 ml/min, detection 214 nm. Compounds are (1) codeine phosphate, (2) ephedrine HCL, (3) theophiline ethyendiamine, (4) atropine sulfate, (5) yohimbine HCl, (6) butylscopolamine Br, (7) papaverine HCl, (6a) butylscopolamine impurity a and (6b) butylscopolamine impurity b.

However, as shown in Figure 35 b, a satisfactory separation of peaks 1, 2, 3 has been achieved by increasing the separation efficiency through connecting two columns together. Although this has doubled the run time in the first place, a subsequent reduction in the run time has been achieved again by applying the flow program mentioned in Table 25 as shown in the chromatogram of Figure 35 c.

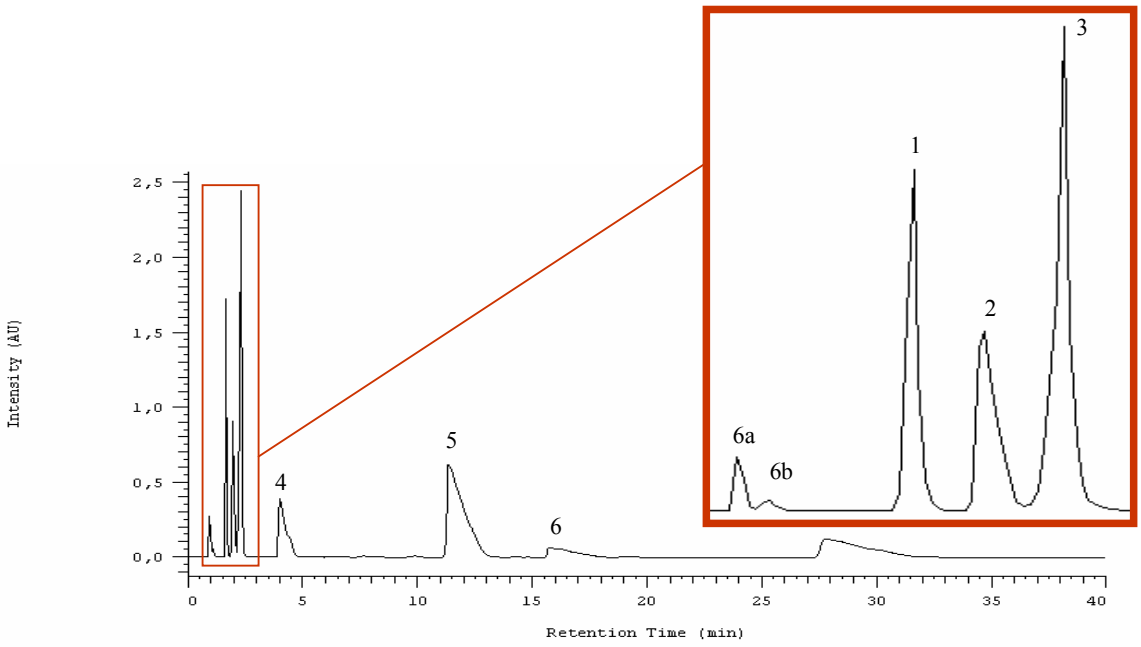


Figure 35 b: Separation of a basic mixture of seven alkaloids. Column:two connected Chromolith Performance Rp-18e, eluent: phosphate buffer 25m*M*, pH 3: methanol (80:20), flow rate: 3 ml/min, detection 214 nm. Peak names as in Figure 35a.

Table 25: The flow program used for the separation of the basic alkaloid mixture of Figure 35c.

Time (min)	Flow rate (ml/min)
0.0	3.0
3.5	3.0
5.0	5.0
5.1	9.0
18	9.0

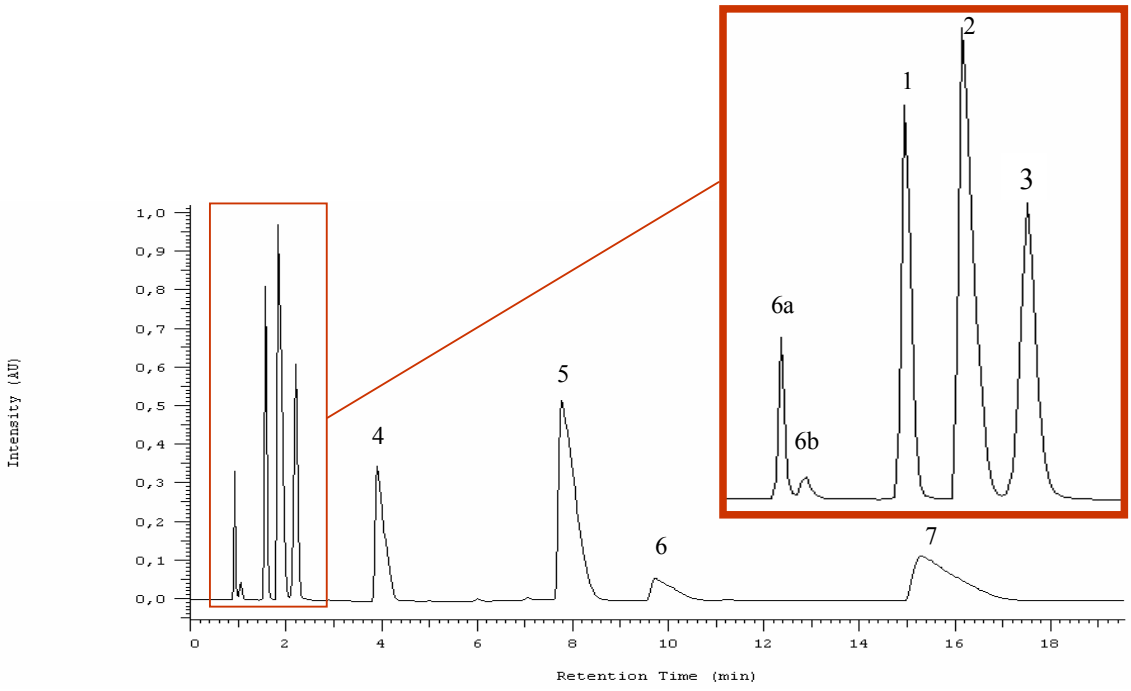
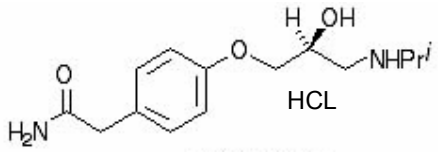


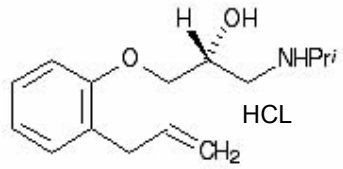
Figure 35 c: Separation of a basic mixture of seven alkaloids. Column: two connected Chromolith Performance Rp-18e, eluent: phosphate buffer 25m*M*, pH 3: methanol (80:20), using the flow program mentioned in Table 25, detection 214 nm. Peak names as in Figure 35a.

3.2 Enantiomeric separation screening results

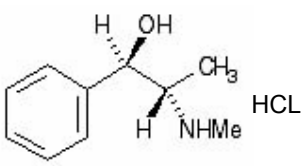
In this work, a methods screening strategy using a set of structurally different cyclodextrins has been applied for enantiomeric separation of various enantiomeric basic drugs. Ten basic enantiomeric compounds have been selected for this screening in the bases of availability and include: atenolol hydrochloride, alprenolol hydrochloride, ephedrine hydrochloride, isoprenaline hydrochloride, methadone hydrochloride, pindolol hydrochloride, promethazine hydrochloride, propranolol hydrochloride, tryptophan, and verapamil hydrochloride. Structures for these tested compounds are given in Figure 36. The method employed the use of a low pH phosphate buffer (pH 2.5). The use of this low pH buffer value has been reported in previous screening strategies [77, 78] and is especially successful for basic enantiomeric drugs as they will be ionized under this acidic condition, thus aiding the solubility and electrophoretic mobility. Seventeen different cyclodextrins have been used in this screening including the three native (α, β, and γ) and derivatized cyclodextrin whether uncharged as hydroxypropyl cyclodextrins or negatively charged as high sulphated, phosphated, carboxymethylated, and succinylated cyclodextrin derivatives. Table 26 and 27 list the screening results for the ten basic racemic drugs using these seventeen different cyclodextrins under different separation modes (normal and reversed polarity). The corresponding resolution value for each successful separation has been calculated. Typical electropherograms obtained for the partial or full successful separation of each drug are shown below in Figures 37-50.



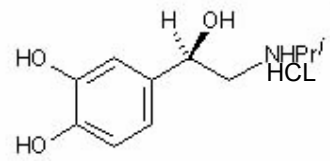
atenolol hydrochloride



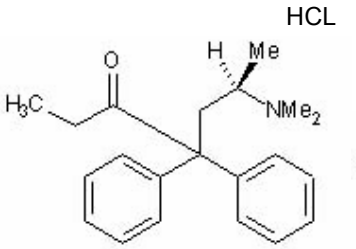
alprenolol hydrochloride



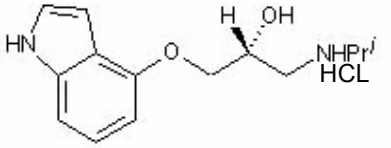
ephedrine hydrochloride



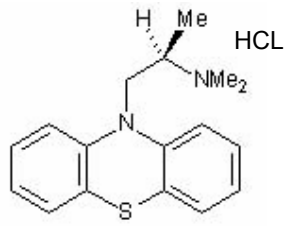
isoprenaline hydrochloride



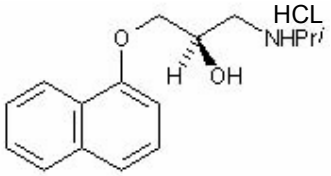
methadone hydrochloride



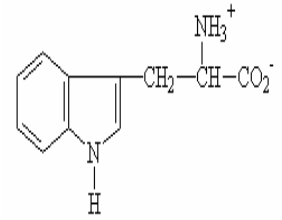
pindolol hydrochloride



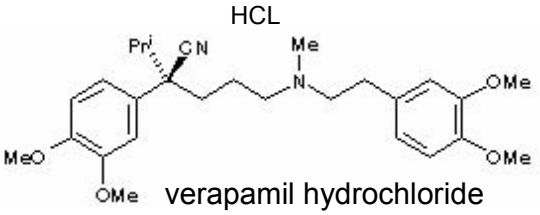
promethazine hydrochloride



propranolol hydrochloride



tryptophan



verapamil hydrochloride

Figure 36: Structure of the ten basic enantiomeric drugs used in this CE screening (pr^i refers to isopropane).

Table 26: Screening result for atenolol, alprenolol, ephedrine, isoprenaline and methadone using different cyclodextrins.

CD Type	Atenolol	Alprenolol	Ephedrine	Isoprenaline	Methadone
α-CD	One Peak (+)	No Peak	No Peak	No Peak	No Peak
β-CD	No Peak	No Peak	No Peak	No Peak	No Peak
γ-CD	No Peak	No Peak	No Peak	No Peak	No Peak
HP-α-CD	No Peak	No Peak	No Peak	No Peak	One Peak (+)
HP-β-CD (Supelco)	No Peak	No Peak	No Peak	No Peak	Two Peaks (+)
HP-β-CD (Sigma)	One Peak (+)	No Peak	No Peak	R_s = 1.1 (+)	Two Peaks (+)
HP-γ-CD	No Peak	No Peak	No Peak	R_s = 1.4 (+)	No Peak
CM-β -CD	No Peak	No Peak	No Peak	R_s = 1.17 (+)	No Peak
CM-γ-CD	No Peak	No Peak	No Peak	R_s = 1.18 (+)	No Peak
HS-α-CD	Peak with 3 tops (+) R_s = 1.37 (-)	R_s = 1.53 (-)	No Peak	No Peak	No Peak
HS-β-CD (Supelco)	Peak with two tops (+) R_s = 0.79 (-)	R_s = 1.92 (-)	Peak with many tops(+)	No Peak	No Peak
HS-β-CD (Sigma)	R_s = 0.67 (-)	No Peak(-) Two peaks one with 2 tops R_s = 1.4	Peak with two tops (+)	One Peak (+) R_s = 5.74 (-)	Two Peaks (+)
HS-γ-CD	R_s = 2.54 (-)	R_s = 1.93 (-)	No Peak	No Peak	No Peak
Phosphated-α-CD	No Peak	Two Peaks R_s = 0.65(+)	No Peak	No Peak	No Peak
Phosphated-β-CD	No Peak	No Peak	No Peak	No Peak	No Peak
Phosphated-γ-CD	No Peak	No Peak	No Peak	No Peak	No Peak
Succinylated - β-CD	No Peak	No Peak	No Peak	No Peak	No Peak

(+) refers to separation under normal polarity

(-) refers to separation under reversed polarity

Table 27: Screening result for pindolol, promethazine, propranolol, tryptophan and verapamil using different cyclodextrins.

CD Type	Pindolol	Promethazine	Propranolol	Tryptophan	Verapamil
α-CD	No Peak	No Peak	No Peak	R_s = 2.45	No Peak
β-CD	No Peak	No Peak	No Peak	No Peak	No Peak
γ-CD	No Peak	No Peak	No Peak	No Peak	No Peak
HP-α-CD	No Peak	No Peak	No Peak	No Peak	One Peak (+)
HP-β-CD (Supelco)	No Peak	No Peak	No Peak	No Peak	Peak with shoulder (+)
HP-β-CD (Sigma)	No Peak	No Peak	No Peak	No Peak	One Peak (+)
HP-γ-CD	No Peak	No Peak	No Peak	No Peak	One Peak (+)
CM-β-CD	Peak with shoulder (+)	No Peak	Peak with two tops (+)	R_s = 0.4	No Peak
CM-γ-CD	No Peak	No Peak	Bad Peak with 2 tops (+)	No Peak	No Peak
HS-α-CD	No Peak	Peak with two tops (-)	No peak (-)	No peak	R_s = 10.2 (-)
HS-β-CD (Supelco)	No Peak	Peak with shoulder (-)	One Peak (-)	No peak	R_s = 1.56 (-)
HS-β-CD (Sigma)	One peak top split (-)	No Peak	One Peak (-)	Two Peaks one small	R_s = 7.42 (-)
HS-γ-CD	Two bad shaped peaks (-)	No Peak	One Peak (-)	No peak	R_s = 6.98 (-)
Phosphated-α-CD	No Peak	No Peak	No Peak	No peak	No Peak
Phosphated-β-CD	No Peak	No Peak	No Peak	No peak	No Peak
Phosphated-γ-CD	No Peak	No Peak	No Peak	No peak	No Peak
Succinylated-β-CD	No Peak	No Peak	No Peak	No peak	Peak with two tops (+)

(+) refers to separation under normal polarity

(-) refers to separation under reversed polarity

For atenolol only peaks with two or many tops were obtained under normal polarity as shown in Figure 37. Under reversed polarity a better separations were obtained ranging from partial to a full base line resolution. However, the best was obtained using HS-γ-CD with a resolution value of 2.57 as shown in Figure 38-d.

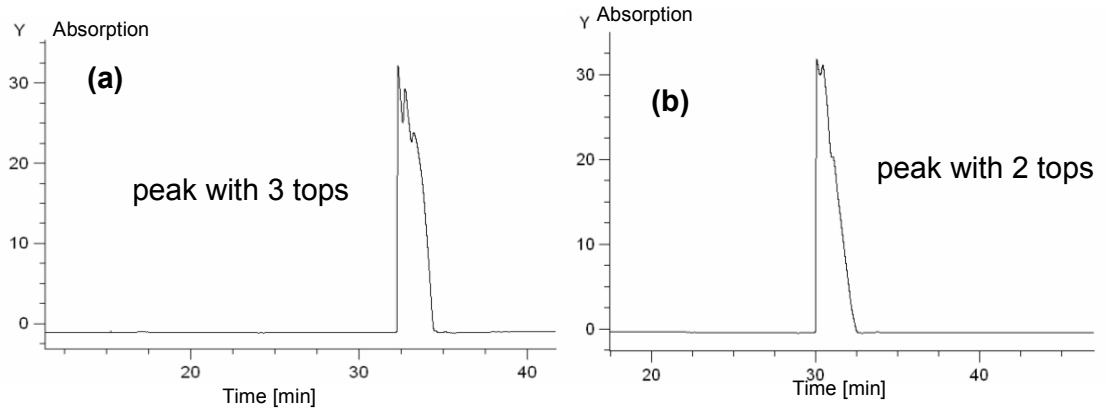


Figure 37: Electropherograms corresponding to the chiral separation of atenolol enantiomers in 50 m*M* phosphate buffer pH (2.5) containing 2% of HS-α-CD in electropherogram (a), and HS-β-CD (Supelco) in electropherogram (b). Injection by pressure, 50 mbar for 0.3 min; temperature 25 °C; capillary, 85 cm, (31 cm to the detector x 50 µm ID); applied voltage, 25 kV (normal polarity); UV detection at 200 nm.

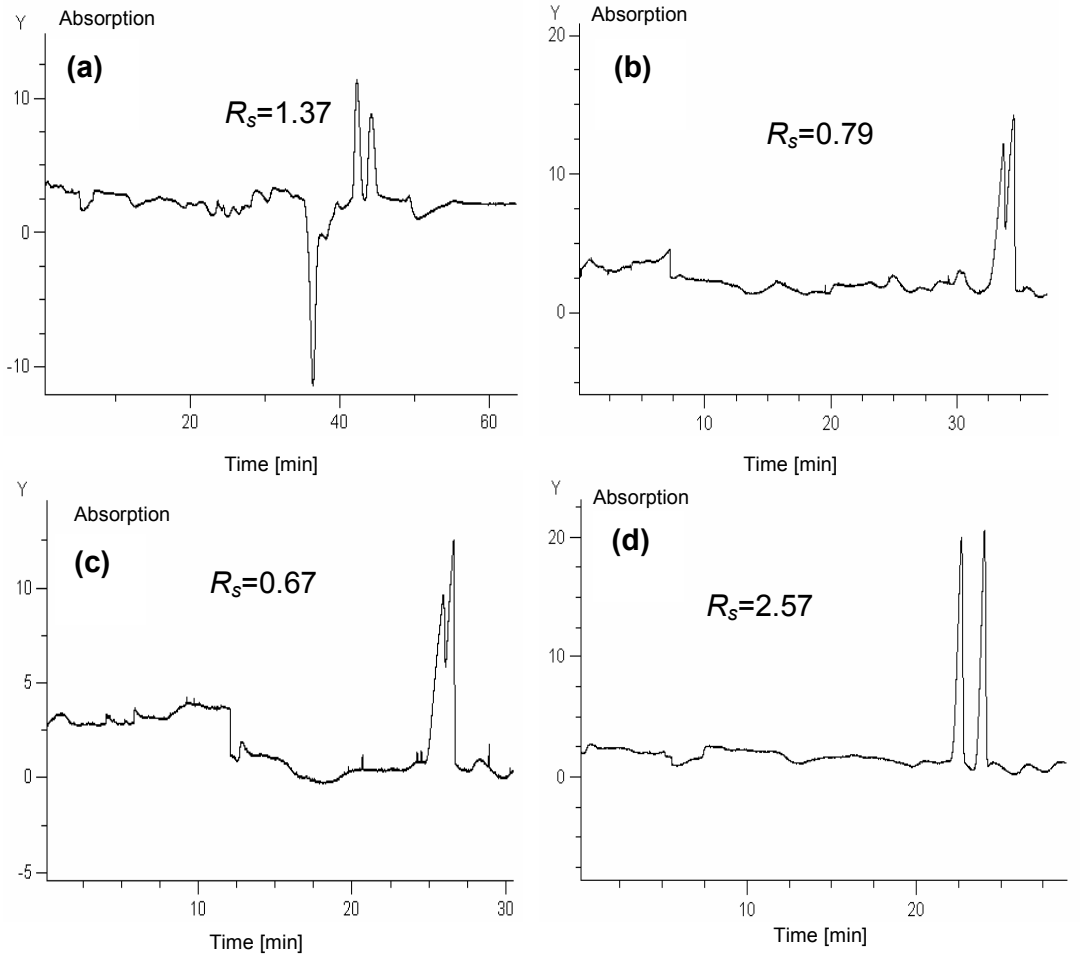


Figure 38: Electropherograms corresponding to the chiral separation of atenolol enantiomers in 50 m*M* phosphate buffer pH (2.5) containing 2% of HS- α –CD in electropherogram (a), HS-β-CD(Supelco) in electropherogram (b), HS- β -CD (Sigma) in electropherogram (c), and of HS- γ -CD in (d). Applied voltage, -25 kV (reversed polarity). Other conditions were as in legend of Figure 37.

For alprenolol normal polarity leads to slight separation (Figure 39). Under reversed polarity two cyclodextrins lead to the best similar separation (Figure 40), one using HS-β-CD (Supelco) (R_s = 1.93 within about 12 min) and the other using HS-γ-CD (R_s = 1.92 within 27 min). It is clear that HS-β-CD (Supelco) will be selected mainly because of the shorter run time.

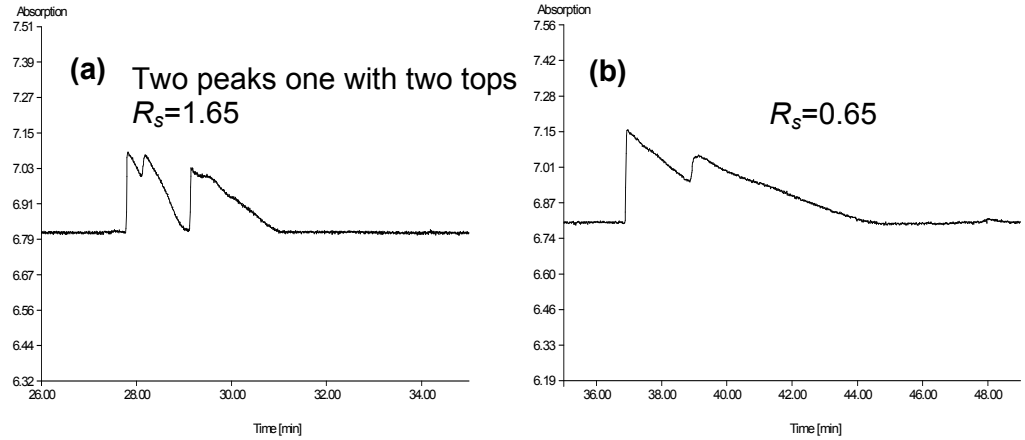


Figure 39: Electropherograms corresponding to the chiral separation of alprenolol enantiomers in 50 m*M* phosphate buffer pH (2.5) containing 2% of HS-β-CD (Sigma) in electropherogram (a), and of phosphated-α-CD in electropherogram (b). Injection by pressure, 50 mbar for 0.3 min; temperature 25 °C; capillary, 62 cm, (48 cm to the detector x 50 µm ID; applied voltage, 15 kV (normal polarity); UV detection at 200 nm.

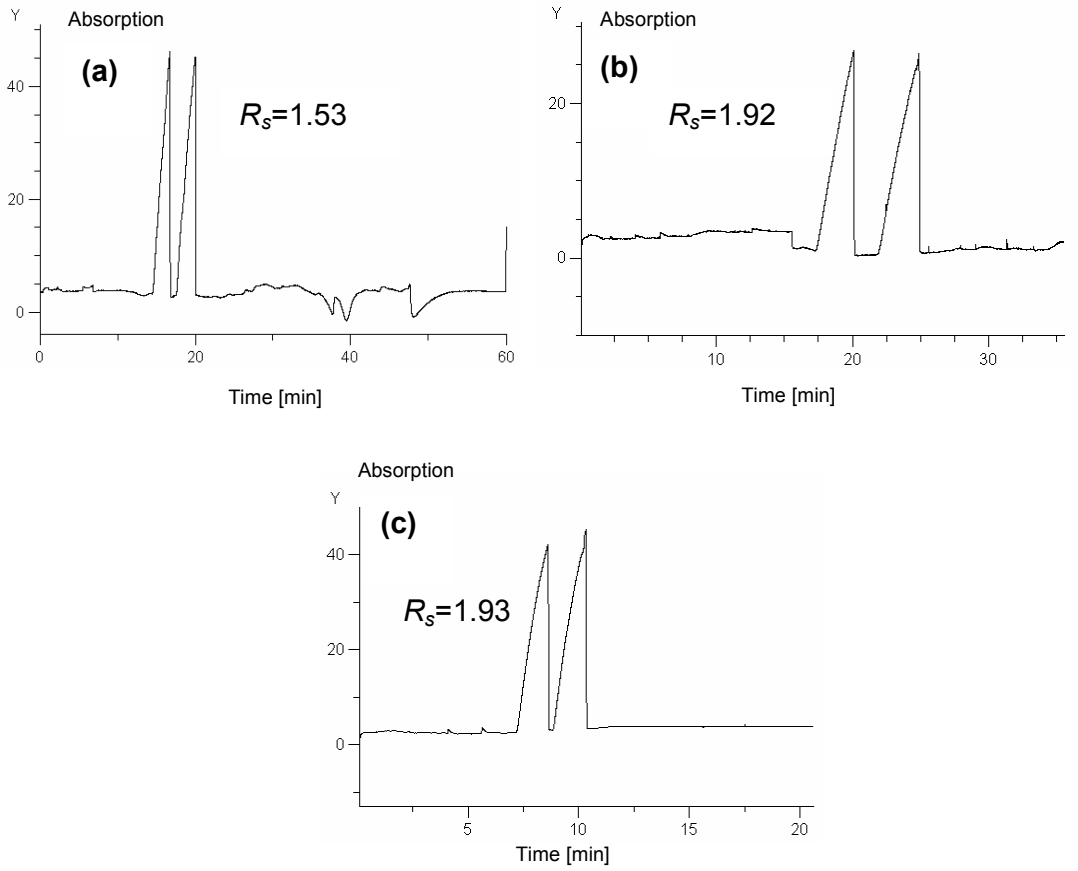


Figure 40: Electropherograms corresponding to the chiral separation of alprenolol enantiomers in 50 m*M* phosphate buffer pH (2.5) containing 2% of HS-α-CD in electropherogram (a), HS-β-CD (Supelco) in electropherogram (b), and of HS- γ -CD in electropherogram (c). Applied voltage -25 kV (reversed polarity). Other conditions were as in legend of Figure 37.

Ephedrine has two chiral centers and so four enantiomers were expected in the product. Unfortunately, the separation was not achieved with any of the tested cyclodextrins under normal and reversed polarity. The only slight separations were obtained using HS-β-CD from Sigma and from Supelco under normal polarity as shown in Figure 41.

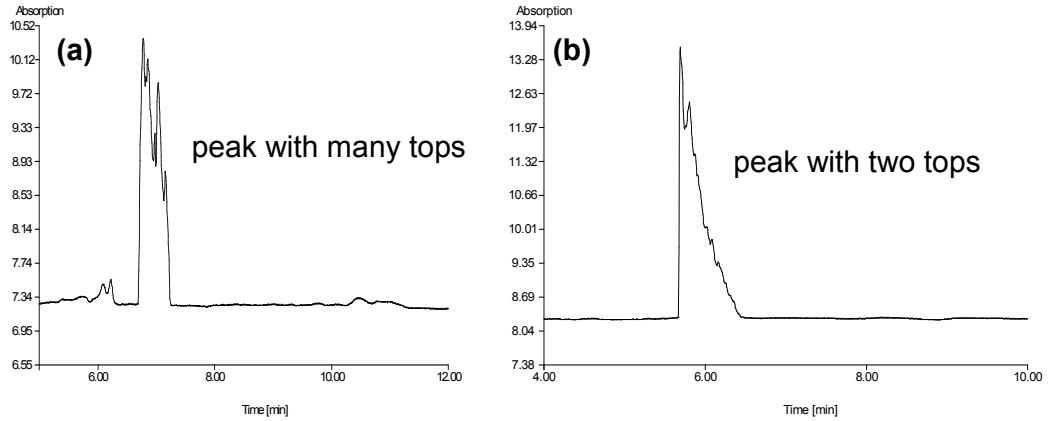


Figure 41: Electropherograms corresponding to the chiral separation of ephedrine enantiomers in 50 m*M* phosphate buffer pH (2.5) containing 2% of HS-β-CD (Supelco) in electropherogram (a), and of HS-β-CD (Sigma) in electropherogram (b). Applied voltage 15 kV (normal polarity). Other conditions were as in legend of Figure 37.

For isoprenaline no separation was achieved with any of the seventeen tested cyclodextrins under normal polarity. Under reversed polarity five different cyclodextrins gave different degrees of enantiomeric separation as shown in Figure 42. The best separation was that obtained using HS-β-CD (Supelco), at which the two isoprenaline enantiomers were separated within 14 min., with a resolution value of 5.74.

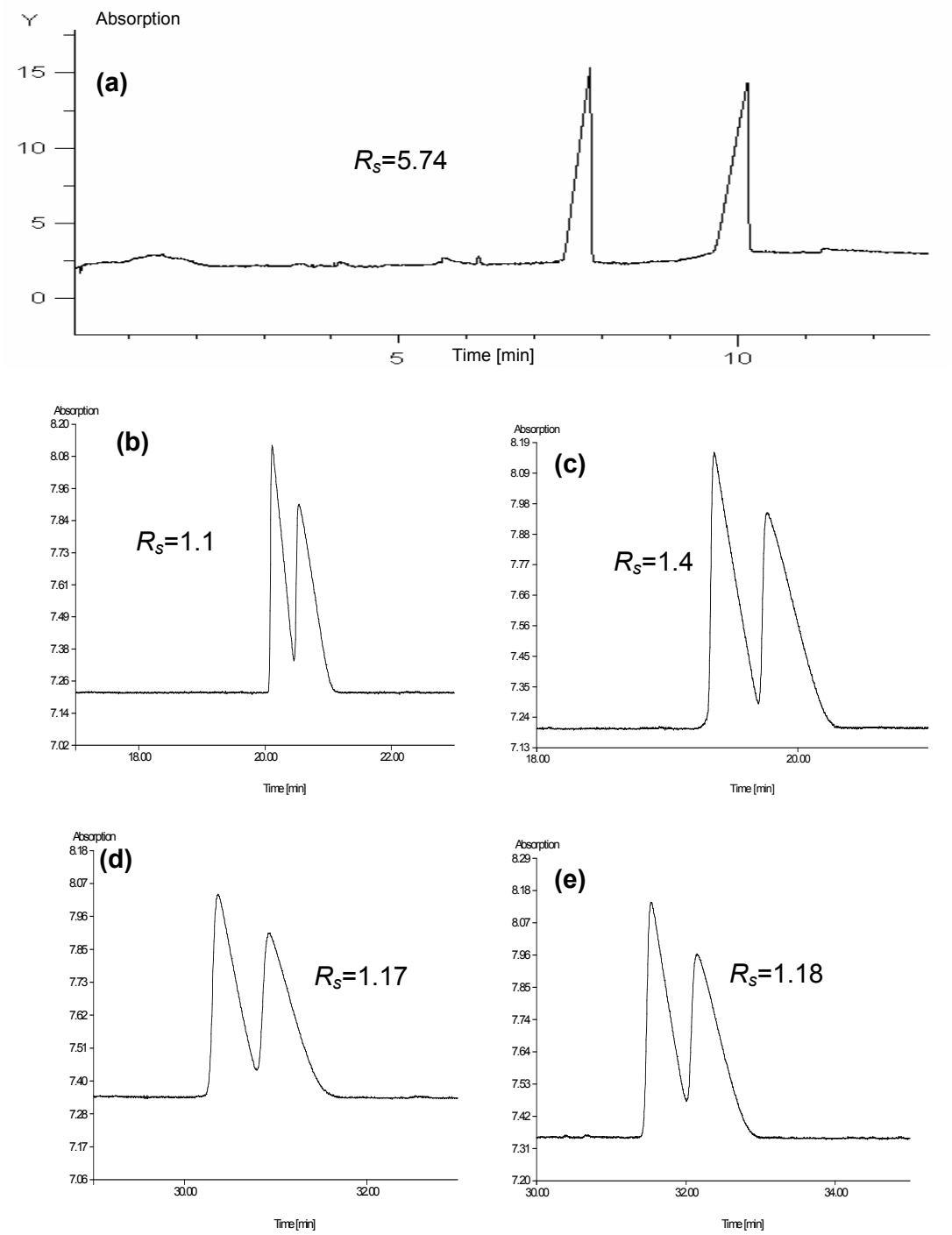


Figure 42: Electropherograms corresponding to the chiral separation of isoprenaline enantiomers in 50 m*M* phosphate buffer pH (2.5) containing 2% of HS-β-CD (Supelco) in (a) HP-β-CD (Sigma) in (b), HP-γ-CD in (c), CM-β-CD in (d), CM-γ-CD in (e). Applied voltage -25 kV (reversed polarity). Other conditions were as in legend of Figure 37.

In the presence of HS-β-CD from Sigma and under normal polarity two peaks were obtained for methadone (Figure 43). However, the second peak is too small in comparable with the first peak and so the separation is doubtful and require peak identification by using the standard individual enantiomers.

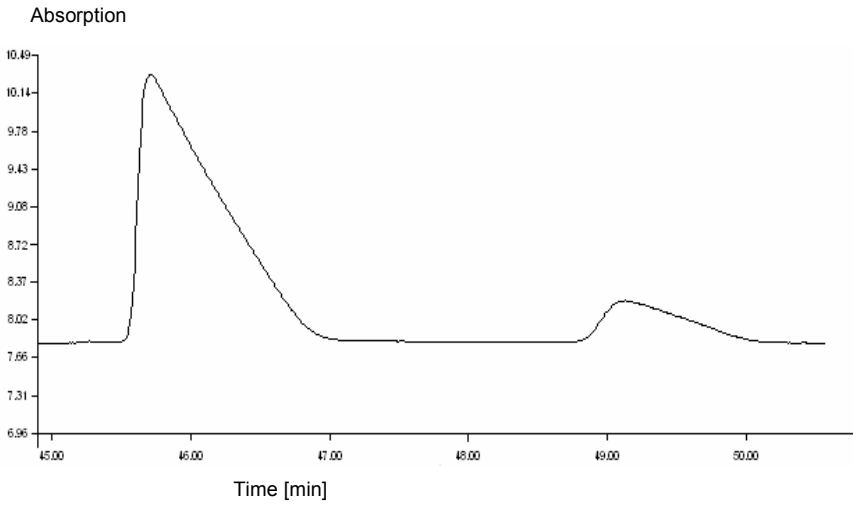


Figure 43: Electropherograms corresponding to the chiral separation of methadone enantiomers in 50 m*M* phosphate buffer pH (2.5) containing 2% of HS-β-CD (Sigma). Applied voltage15 kV (normal polarity). Other conditions were as in legend of Figure 39.

For pindolol a peak with shoulder has been obtained using CM-β-CD under normal polarity. This indicates a slight beginning of separation (Figure 44). While under reversed polarity the best obtained result was with HS-γ-CD were two bad shape peaks have been obtained as shown in Figure 45, however this is still considered as a poor initial conditions and should require intensive method optimization.

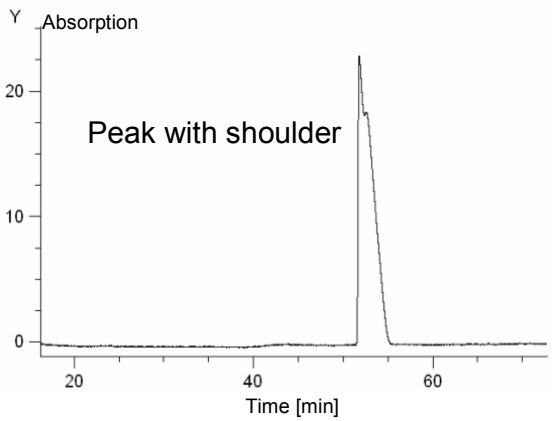


Figure 44: Electropherograms corresponding to the chiral separation of pindolol enantiomers in 50 m*M* phosphate buffer pH (2.5) containing 2% of CM-β-CD. Applied voltage, 10 kV (normal polarity polarity). Other conditions were as in legend of Figure 37.

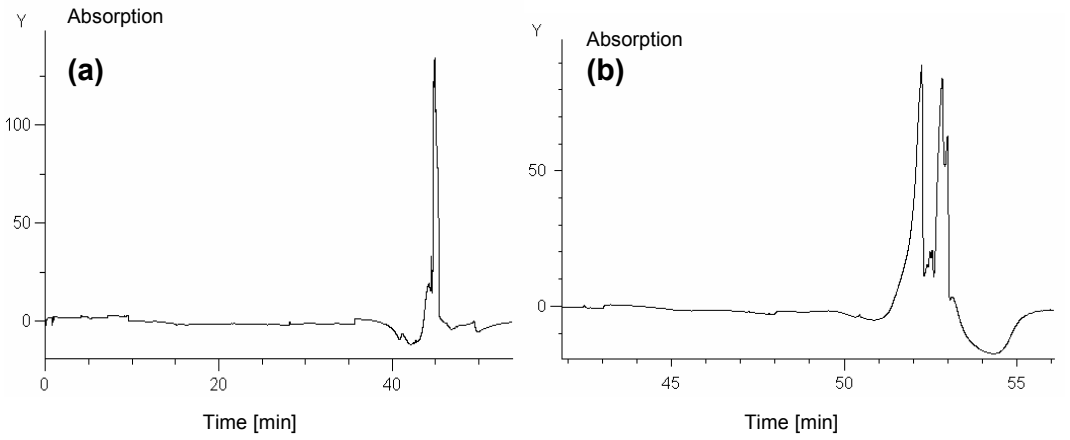


Figure 45: Electropherograms corresponding to the chiral separation of pindolol enantiomers in 50 m*M* phosphate buffer pH (2.5) containing 2% of HS-β-CD (Sigma) in electropherogram (a), and of HS-γ-CD in (b). Applied voltage, -25kV (reversed polarity). Other conditions were as in legend of Figure 37.

In the presence of HS-α-CD and under reversed polarity a peak with two tops was obtained for promethazine within a relatively short analysis time as shown in Figure 46-a.

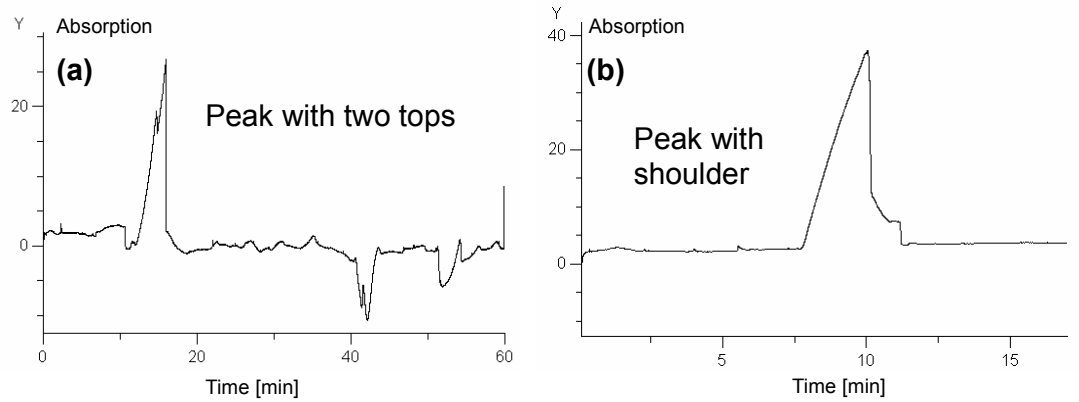


Figure 46: Electropherograms corresponding to the chiral separation of promethazine enantiomers in 50 m*M* phosphate buffer pH (2.5) containing 2% of HS-α-CD in electropherogram (a), and of HS-β-CD (Supelco) in (b). Applied voltage, -25kV (reversed polarity). Other conditions were as in legend of Figure 37.

For propranolol no separation was obtained with any of the cyclodextrin under reversed polarity. Applying the normal polarity slight separations that involve peaks with shoulders were obtained with CM-β-CD and CM-γ-CD (Figure 47), however the better peak shape was that obtained with the CM-β-CD and so will be the one selected as initial separation condition. As can be seen in Figure 48, good baseline separation was obtained for tryptophan with both α-CD and CM-β-CD. However, the

best resolution value was obtained with the native cyclodextrin α-CD that achieved a resolution value of 2.45 and in a total analysis time of about 25 min.

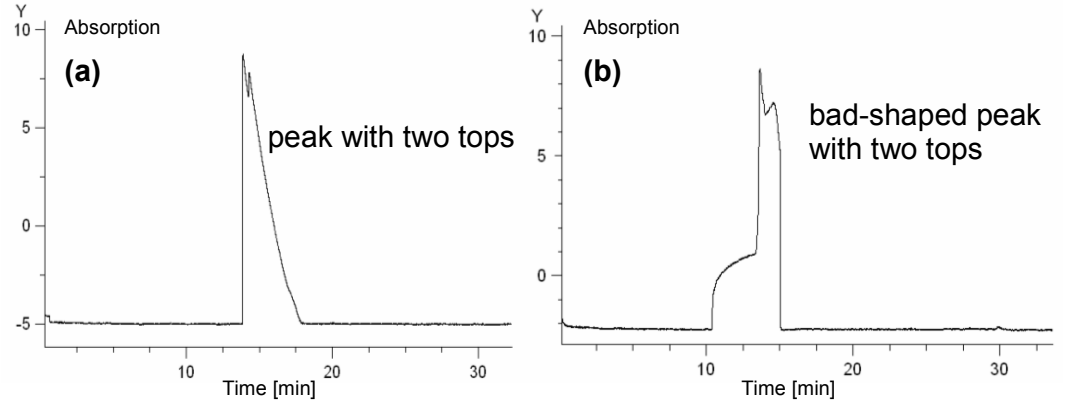


Figure 47: Electropherograms corresponding to the chiral separation of propranolol enantiomers in 50 m*M* phosphate buffer pH (2.5) containing 2% of CM-β-CD in electropherogram (a), and of CM-γ-CD in electropherogram (b). Applied voltage, 10 kV (normal polarity). Other conditions were as in legend of Figure 37.

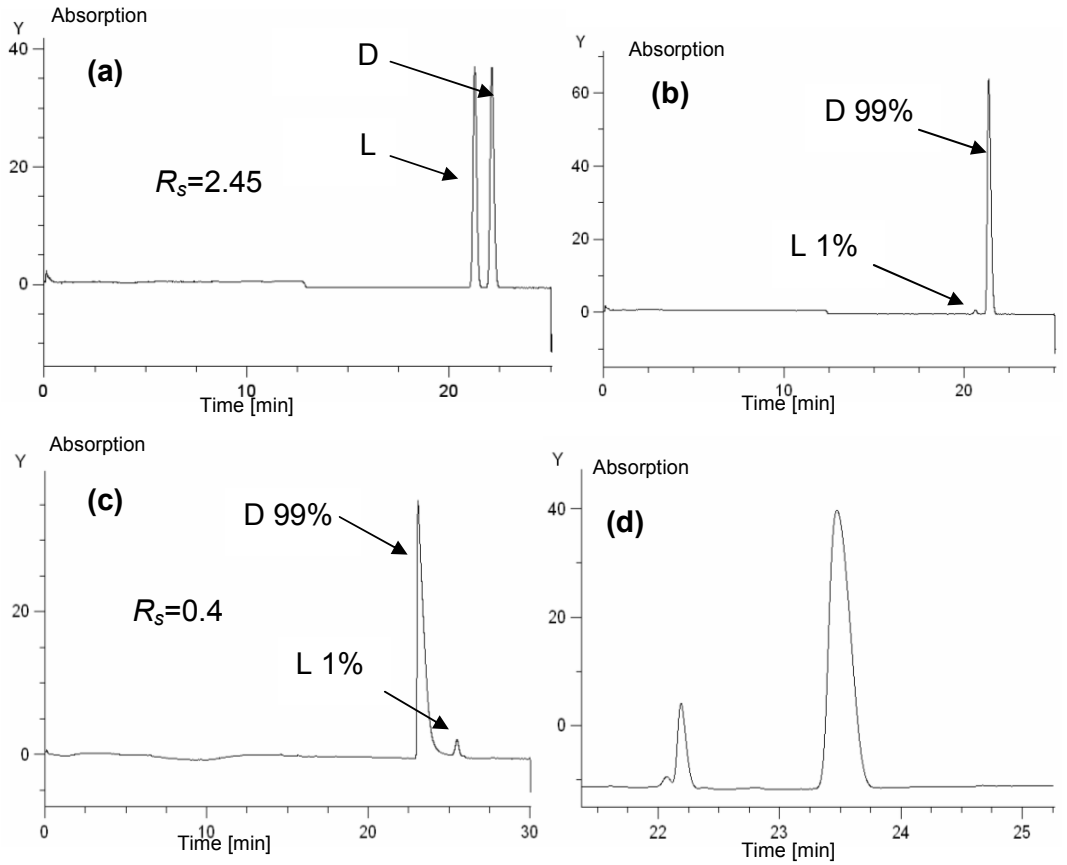


Figure 48: Electropherograms corresponding to the chiral separation of tryptophan enantiomers in 50 m*M* phosphate buffer pH (2.5) containing 2% of α-CD in electropherogram (a) and (b), and of CM-β-CD in electropherogram (c), and of HS-β-CD (Sigma) in electropherogram (d). Applied voltage, 10 kV (normal polarity). Other conditions were as in legend of Figure 37.

Under normal polarity slight separation mainly peak with shoulder was obtained for verapamil using HP-β-CD from Supelco (Figure 49). On the other hand, four sets of suitable initial conditions were obtained for verapamil under reversed polarity (Figure 50). High resolution was achieved using HS-α-CD, HS-β-CD (Sigma) and HS-γ-CD. The best overall resolution for verapamil (R_s = 10.19) was achieved by HS-α-CD within a total migration time of about 23 min. The use of HS-β-CD (Sigma) gave a lower resolution within the same migration time (R_s = 7.42). However HS-γ-CD gave a resolution of (R_s = 6.98) but within a shorter time of 15 min. Then, the choice of the best cyclodextrin for the separation of verapamil will depend on which is more important for the analysis goal, time or high resolution. Short analysis time is more important when the method is intended for the separation and/or quantitation of individual enantiomers when they present in a comparable concentrations. The short time will then be more suitable for a series of runs in the automated instruments to save the analysis time. However, if the method is intended to be used for trace enantiomer (enantiomeric impurity determination) then it is more important to have a high resolution than to look to the analysis time. The high resolution will prevent the peaks overlap between the main enantiomers and impurity enantiomer. This becomes clearly important when sample volume or mass overload is used in order to reach a lower limit of quantitation for the impurity peak.

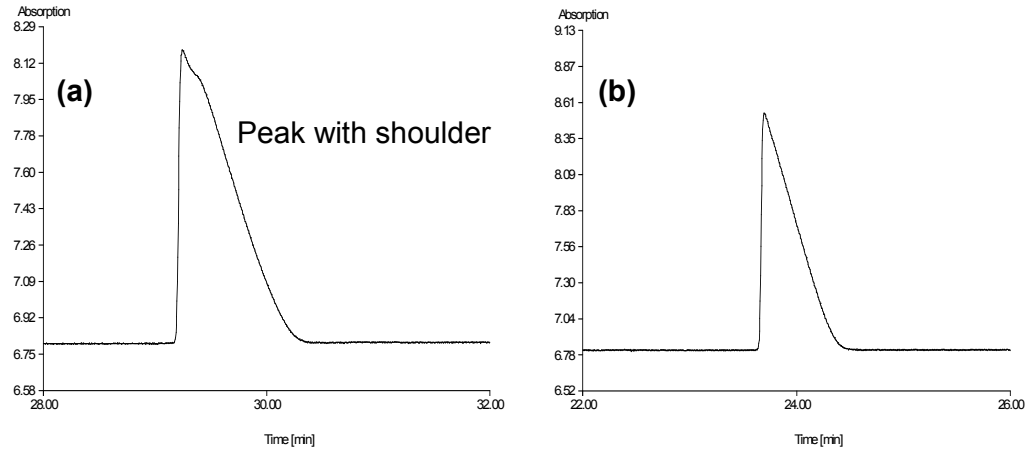


Figure 49: Electropherograms corresponding to the chiral separation of verapamil enantiomers in 50 m*M* phosphate buffer pH (2.5) containing 2% of HP-β-CD (Supelco) in electropherogram (a), and of HP-β-CD (Sigma) in electropherogram (b). Applied voltage, 25 kV (normal polarity). Other conditions were as in legend of Figure 39.

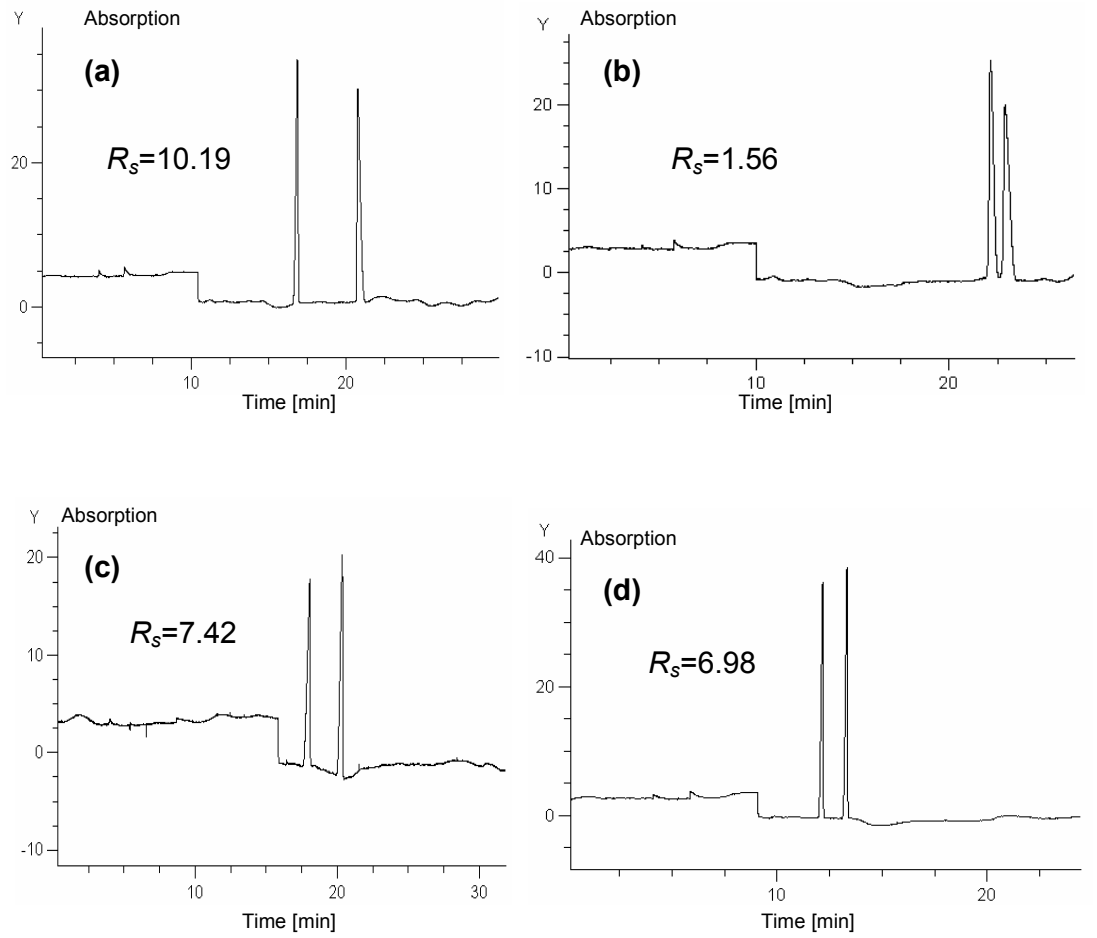


Figure 50: Electropherograms corresponding to the chiral separation of verapamil enantiomers in 50 m*M* phosphate buffer pH (2.5) containing 2% of HS-α-CD in electropherogram (a), and of HS-β-CD (Supelco) in electropherogram (b), and of HS-β-CD (Sigma) in electropherogram (c), and of HS-γ-CD in electropherogram (d). Applied voltage, -25 kV (reversed polarity). Other conditions were as in legend of Figure 37.

4. Discussion

4.1 HPLC part

4.1.1 Transferred methods

Precision was slightly better on monolithic columns than on the conventional columns possibly due to the better peak shape and reduced baseline noise that leads to more precise integration. The selected methods include different ratios of organic modifier in the mobile phase: 2% in the pilocarpine method, 26% in the insulin method, 45% in the glibenclamide method and 60% in the propranolol method. This will probably give a more representative evaluation of precision. Because repeatability could be affected by the percentage of organic modifier in the mobile phase, but this was not found in this study. When higher flow rates were applied on monolithic columns there was some loss in resolution. A flow rate of 4 ml/min was selected for precision studies, as it provides the smallest analysis time with a baseline resolution (R_s values higher than 2) for all investigated methods.

Methods for the small drug molecules pilocarpine, propranolol, glibenclamide and glimepiride with their degradation compounds or related products were successfully transferred without any modification. For insulin, optimizing selectivity by slightly decreasing the percentage of organic modifier in the mobile phase was sufficient for a good resolution on the monolithic column. The tiny decrease of organic modifier has a dramatic effect on the retention time of insulin (more than doubling them) and desamido-insulin whereas the retention times of the small molecules phenol and cresol were kept nearly unchanged. It is not clear why the insulin method was not successfully transferred from the conventional particle-packed to the monolithic column under the same chromatographic conditions. However, one must keep in mind that the mechanism by which polypeptides interact with the reversed-phase surface is a bit different as that for small drug molecules. The separation of small molecules involves continuous partitioning of the molecules between the mobile phase and the hydrophobic stationary phase. Polypeptides, however, are too large to completely partition into the hydrophobic phase; possibly they adsorb to the hydrophobic surface after entering the column. They then desorb and interact only slightly with the surface as they elute down the column. Based on this mechanism, the improved mass transfer of monolithic over conventional columns will highly accelerate the elution of polypeptides after desorption.

Peak tailing with monolithic columns was reported in some previously mentioned studies [79, 80]. However, in many other works with monolithic columns a minimal or no peak tailing was reported [81-83]. In fact, peak tailing in reversed phase HPLC is particularly prevalent when separating basic compounds. It causes a number of problems including lower resolution, reduced sensitivity and poor precision and quantitation. There are many reasons for peak tailing including injecting sample in a solvent that is significantly stronger than the mobile phase, sample mass overload and a void in the column packing bed. However, the common cause of peak tailing in reversed phase HPLC is the secondary retention. This occurs when an ion-exchange interaction takes place between a positively charged solute (ammonium) and an acidic silanol on the surface of the silica stationary phase support particles. It is observed most often when using HPLC columns packed with stationary phases that have significant silanol activity. It is usually worse at neutral pH (6 - 8) than at acidic pH (< 3). The less the silanol activity the stationary phase exhibits the less the peak tailing. In our investigations all the tested methods were successfully done using a mobile phase buffer in the acidic range (pH around 3) therefore no strong tailing was observed.

As expected, the total analysis time was reduced to about a quarter at a flow rate of 4 ml/min using monolithic columns. A diagram for the percent reduction in analysis time for successfully transferred methods from conventional to monolithic columns is shown in Figure 51.

The lower limits of detection and quantitation obtained by the monolithic columns are partly due to the lower background noise obtained with these columns. In this evaluation of monolithic Chromolith Performance columns the polypeptide insulin was also investigated. Larger molecular weight proteins were not investigated because the applicability of reversed phase silica for the quantitation of large molecular weight portions suffers from the problem of adsorption and the loss of results repeatability. Few papers were found in the literature suggesting reversed phase chromatography for the quantitation of proteins [84-86]. Furthermore, silica based monolithic columns are particularly suited for the separation of small molecules, such as drug candidates and peptides, while the polymer monolith are generally preferable for larger molecules such as proteins, nucleic acid, and synthetic polymers. In a comparison between conventional high performance liquid chromatography (HPLC) and ultra

performance liquid chromatography (UPLC) carried out by the Water Company, they have reported the solvent consumption as a disadvantage of using monolithic columns [87].

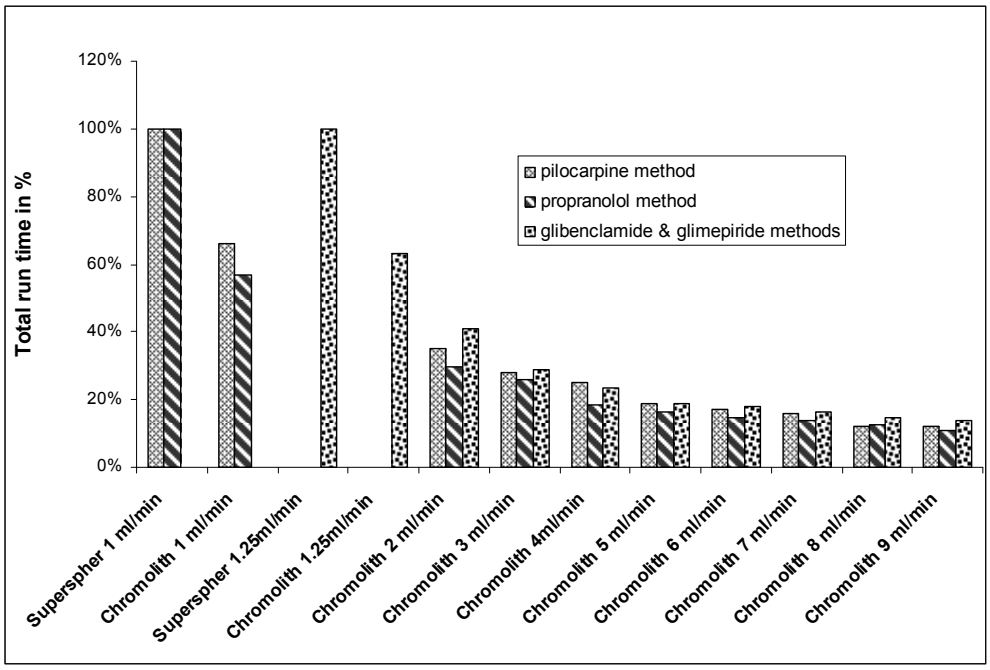


Figure 51: Reduction in total run time for the three successfully transferred methods.

According to our work, the increase in solvent consumption of monolithic column**s** at high flow rates was totally compensated by the decrease in the chromatographic run time (Figure 52). The high permeability of the monolithic columns was evidenced by a flow rate of 9 ml/min generating a total system back pressure of less than 240 bar in all of the four tested methods. In comparison, the conventional column packed with 4 µm particles, reached a maximum backpressure of about 400 bar when it was operated at a flow-rate of 3.5 or 4 ml/min depending on the composition of the used mobile phase (Figure 53).

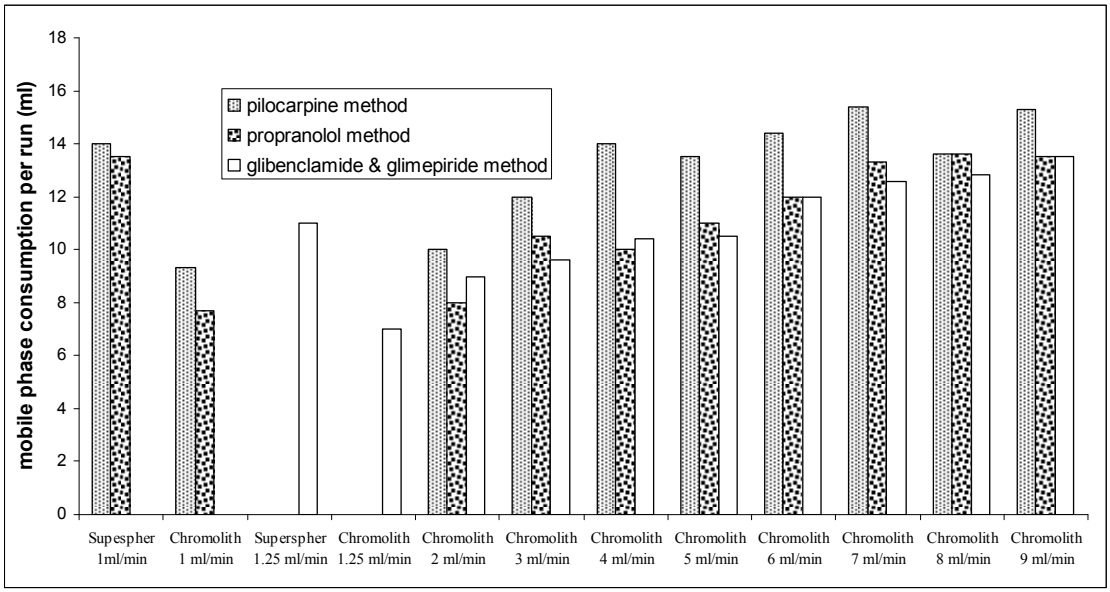


Figure 52: Comparison between Superspher and Chromolith performance columns in mobile phase consumption, during the run time for the three successfully transferred methods.

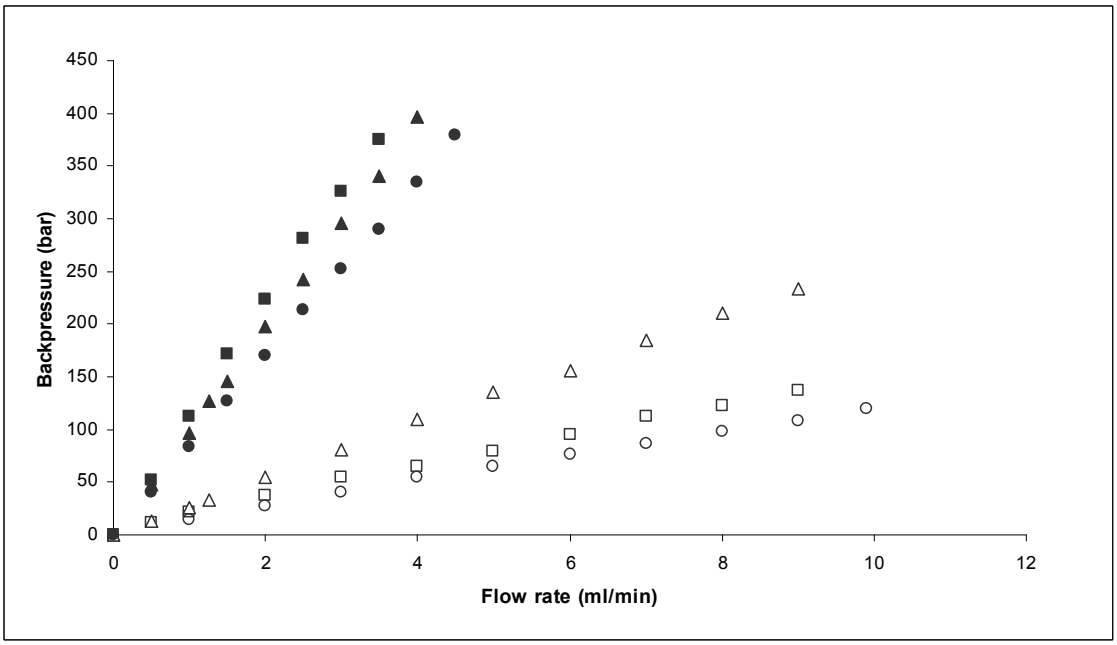


Figure 53: Plot of system backpressure against flow rate for the three successfully transferred methods. Pilocarpine (squares), propranolol (triangles) and glibenclamide & glimepiride (circles). Closed and open signs refer to values on conventional and monolithic columns, respectively.

At a flow rate of 1 ml/min backpressure is about five times smaller on a monolithic (Chromolith Performance RP-18e) column than on a conventional (Superspher RP 18e) column (Figure 54).

Flat curves were obtained for plate height against the flow rates of mobile phases for the four tested methods. This indicates that monolithic columns can be operated at high flow rates with only small decrease in efficiency. The separations on monolithic columns were performed with shorter run time, better peak symmetry and the same or better resolution compared to the conventional column, under the same chromatographic conditions. In a conventional Superspher column more time was required to re-equilibrate or to wash the stationary phase at a flow of 1ml/min (approximately 30 min).

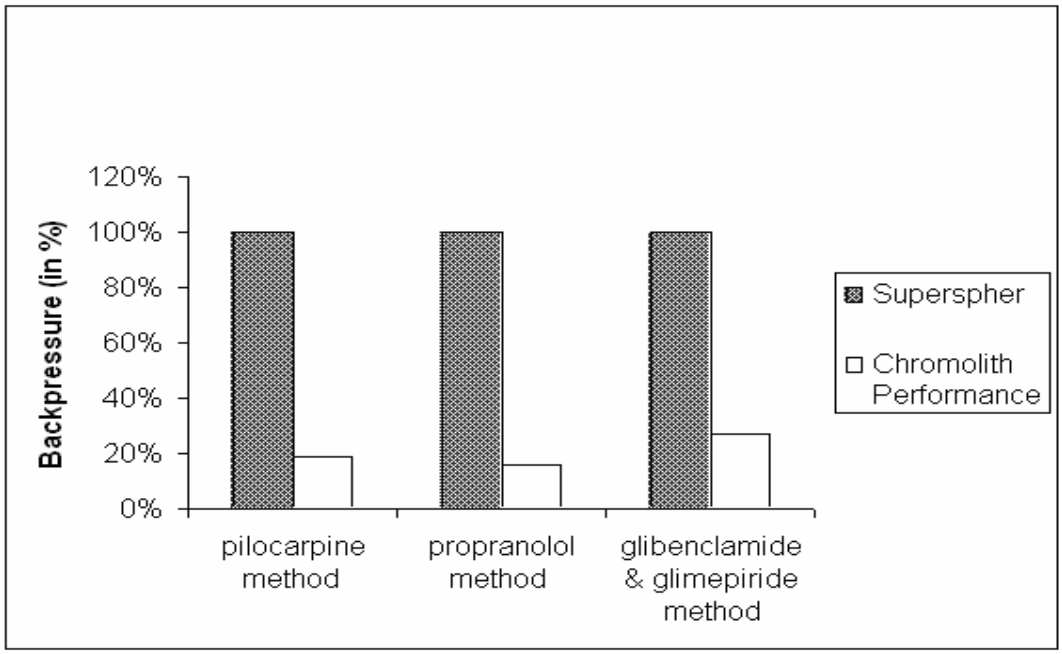


Figure 54: Reduction in system backpressure for the three successfully transferred methods from Superspher to Chromolith Performance columns under the same flow rate.

In contrast, the time required to re-equilibrate or wash the monolithic columns until a smooth baseline is obtained was 6 times shorter (re-equilibrium time was about 5 min at a flow of 6 ml/min) due to the higher flow rates which have been used for equilibrium. These favourable flow rates are possible using monolithic silica with its typical structure and distribution of mesopores.

4.1.2 High speed analysis with flow programming

An important parameter to be considered in a chromatographic separation is the duration of the analysis. Accordingly, fast HPLC methods are important to improve productivity e.g. in pharmaceutical analysis. This work presents a fast method for simultaneous separation and determination of glimepiride, glibenclamide and two related substances by reversed phase liquid chromatography. The data presented demonstrates explicit advantages of monolithic columns for the fast HPLC analysis of drugs.

The application of high velocity isocratic elution with monolithic column was limited by the loss of resolution between the closely related peaks of related products a and b (see Figure 25). In such cases, further reduction in chromatographic run time could be achieved by the application of gradient elution of mobile phase or flow rate programming. In gradient elution, the solvent polarity (composition) is continuously varied or stepped. Two high pressure liquid pumps and a system for mixing and degassing the mobile phase must be used. Furthermore, the HPLC gradient mixers must provide a very precise control of solvent composition to maintain a reproducible gradient profile. The use of flow rate gradient (flow-rate programming) in HPLC separations involves a stepwise increase in the flow rate using one pump according to a defined flow program. As an important advantage of flow rate program over gradient elution of mobile phase, equilibration of the system is not required after each separation [88-96]. This is important to achieve fast analysis of a series of samples. Flow rate programming is more suitable for monolithic than conventional particle-packed columns due to higher permeability and lower backpressure. Instrumentation failure due to high column back pressure usually occurs when flow programming is applied on conventional particle-packed columns [97, 98]. The use of flow programming eliminates the need of re-equilibrium time between successive runs which is required in case of mobile phase gradient. The 4 peaks were eluted within less than 80 second showing that the method can be used as an efficient rapid method for series drug analysis.

Usually, the increase in column temperature in reversed phase HPLC leads to a decrease in peak broadening and retention time [99-101]. Accordingly high column temperature in combination with a flow program seems to be promising for significant reduction in analysis time. Results showed that the application of high column

temperature in combination with flow programming in monolithic column did not lead to an additional reduction in analysis time.

Even though, conventional and monolithic silica columns are stable over a similar temperature range up to 45°C. Increasing temperature is problematic with monolithic columns because they are packed in insulating PEEK [poly(ether ethyl ketone)], indicating the importance of applying other type of heating in the column oven.

RSD% of the interday and the intraday repeatabilities for both retention times and peak areas for the four analyzed compounds were less than 1.2%. The method showed good linearity and recovery. The short analysis time makes the method very valuable for quality control and stability testing of drugs and their pharmaceutical preparations.

4.1.3 Method development

The parameters that control separation in monolithic silica columns are the same for conventional silica columns and for reversed phase HPLC separation in general. However the effect of certain parameters on the separation may differ. For example parameters as flow rate play a more important role in monolithic silica columns than in conventional particle packed columns. On the other hand, parameters as organic modifier or pH of the mobile phase play nearly the same role for the two column types.

Flow rate is an important separation parameter that makes a great difference between method development on conventional and monolithic silica columns. The high permeability offered by the high porosity of the column allows the use of high flow rates without the development of a significant backpressure. A standard LC method can be converted to a fast LC method on monolithic columns, only by increasing the flow rate. In monolithic columns, the increase in flow rate does not lead to substantial losses in resolution because of the low mass transfer resistance compared to conventional particle-packed columns (See Figure 27 section 3.1.1.3 for plate height). This provides a time saving method with minimal loss of resolution. The reduction in analysis time could be achieved either by applying a high isocratic flow rate or using flow rate programming.

Monolithic RP-18e column has been used as a stationary phase which is an important parameter to affect separation. However, it was still possible to change the column length by connecting several columns together using a column coupler. Due

to the high column porosity the added column backpressure was still acceptable and the prolonged analysis time was compensated by flow programming. Increasing column length was particularly important for complex basic mixtures. Usually these require the use of high pH mobile phases which is not suitable with silica columns.

As with conventional columns, decreasing the percentage of organic modifier will decrease the elution strength, increase the retention time and improve resolution. Acetonitrile and methanol have been selected as the first choice organic modifiers. Tetrahydrofuran (THF) which is a possible solvent in reversed phase chromatography has been avoided due to its incompatibility with the long term use with PEEK tubing of the monolithic column in addition to its well known disadvantages as high absorbance and reactivity with oxygen.

Even though, too much water in the mobile phase can collapse the bonded phase, we have obtained a reliable separation for pilocarpine analysis on monolithic column with as little as 2% methanol in the mobile phase (See Figure 23 section 3.1.1.3 for pilocarpine chromatograms).

As for conventional silica columns, the pH stability range for monolithic columns range from pH 2.0 to pH 7.5. For acidic compounds one will probably succeed to achieve a full resolution between the individual compounds using an acidic mobile phase. They will be unionized at pH 2 and thus better retained with the possibility to obtain the ionizable form for some component by raising the pH in the acidic range to improve the selectivity. At pH value of more than ± 1.5 of the pK_a the compound will be either almost completely ionized or unionized [102].

For basic compounds it is also better to achieve separation in the acidic or mild pH ranges because of two reasons: first, because the silica backbone is soluble at high pH value. Second reason is to decrease secondary interaction between basic compounds and ionized silanol groups of the silica column which leads to extensive peak tailing. This effect is minimized at acidic pH at which silanols are nonionized.

However, some basic compounds have high pK_a value (as some alkaloids), it could be difficult to obtain full resolution between the individual components without using a high pH mobile phase, which is in turn problematic with silica based RP-18 columns for the above discussed reasons. In this case it might be an advantageous strategy to skip back to mild pH conditions and to improve only a partial separation of peaks by column coupling. The increase in column length will gain better resolution. The

increased run time due to increased column length could then be reduced by applying a proper flow rate program.

Increasing temperature decreases retention time and band spacing. However, this effect is small, and furthermore is greater on ionic than on neutral samples. At least it is better to work under constant temperature to maintain constant retention and resolution especially for ionic samples [102]. Conventional and monolithic columns are stable over the same temperature range up to 45 °C. However, it is not possible to use high temperature with monolithic columns as they are insulated with the packing material of PEEK [Poly (ether ethyl ketone)]. To overcome this problem, the mobile phase should be heated in a water bath and mobile phase lines covered with insulator. At the same time a stainless steel capillary connected to the column inlet should be placed with the column in the oven. This will insure a certain temperature inside the column in spite of the insulating column tube. Furthermore, other heating strategies as using microwaves could be tried in column ovens.

In addition to the above discussed parameters, having a high porosity and low back pressure offer the advantages for decreasing washing and re-equilibrium times for monolithic columns during method development. This could be done by applying high flow rates for re-equilibrium and washing up to 9 ml/min for 3 minutes (See also Chart 2 section 3.1.3.1). Furthermore this provides less care about the column blockage by contaminants. Because of the density of monolithic columns is much lower, the loadability of a conventional column of the same size is much higher [103]. Accordingly, one should take care not to inject a too large sample weight or volume as it is possible for the mass of the sample to overload the column.

4.2 Enantiomeric separation discussion

Method development in chiral separation is complicated because of the many chiral selectors available. A simple separation strategy has been used for the rapid screening of basic enantiomeric drugs. The aim was the rapid evaluation of the best cyclodextrin for the separation of each of the ten basic enantiomeric drugs. An acidic phosphate buffer pH 2.5 100 m*M* has been used to have the basic enantiomers in the ionized form as positively charged species. This will probably provide more interaction with CD specially the negatively charged one. However, the buffer concentration was then reduced from 100 to 50 m*M* to reduce the current and so the capillary temperature specially because the addition of ionic CDs to the buffer solution increases the current intensity and it is recommendable to work at low current intensities (< 100 µA).

Screening was started with the native neutral CDs. Experiments showed that the fast majority of successful separations have been achieved with the negatively charged cyclodextrins under reversed polarity. Under reversed polarity mode the anode is at the outlet (in contrast to normal polarity mode), keeping in mind the conditions of suppressed electro-osmotic flow (due to the acidic buffer pH 2.5) the negatively charged cyclodextrins have strong electrophoretic mobility toward the positive electrode (anode). If the enantiomers interact with these negatively charged cyclodextrins, they will be swept toward the anode regardless of charge state. Now keeping in mind also that the enantiomers of the basic drugs are positively charged under this low pH buffer they are more likely to interact with this negatively charged cyclodextrin and then the cyclodextrin drug complex will be attracted toward the anode at the outlet and pass the detection window. Figure 55 shows representative schemes for the mechanism of separation of basic enantiomeric drugs inside the capillary using negatively charged cyclodextrin under suppressed EOF by low pH value in normal and reversed polarity modes. While neutral compounds interact normally with the hydrophobic cavity of this negatively charged cyclodextrin, these basic compounds will be strong cations at low pH interacting both with the hydrophobic cavity and ionically with the negatively charged groups of the cyclodextrin. Identification of individual enantiomers peaks after separation has been done before method optimization using standard pure enantiomers. It has been noted that the same type of derivatized cyclodextrin from different commercial source gave

a different separation result under the same conditions. For example in Figure 38 HS-β-CD from Supelco gave better resolution for the separation of atenolol than HS-β-CD from Sigma (R_s value = 0.79 using HS-β-CD from Supelco compared to R_s value = 0.67 using HS-β-CD from Sigma).

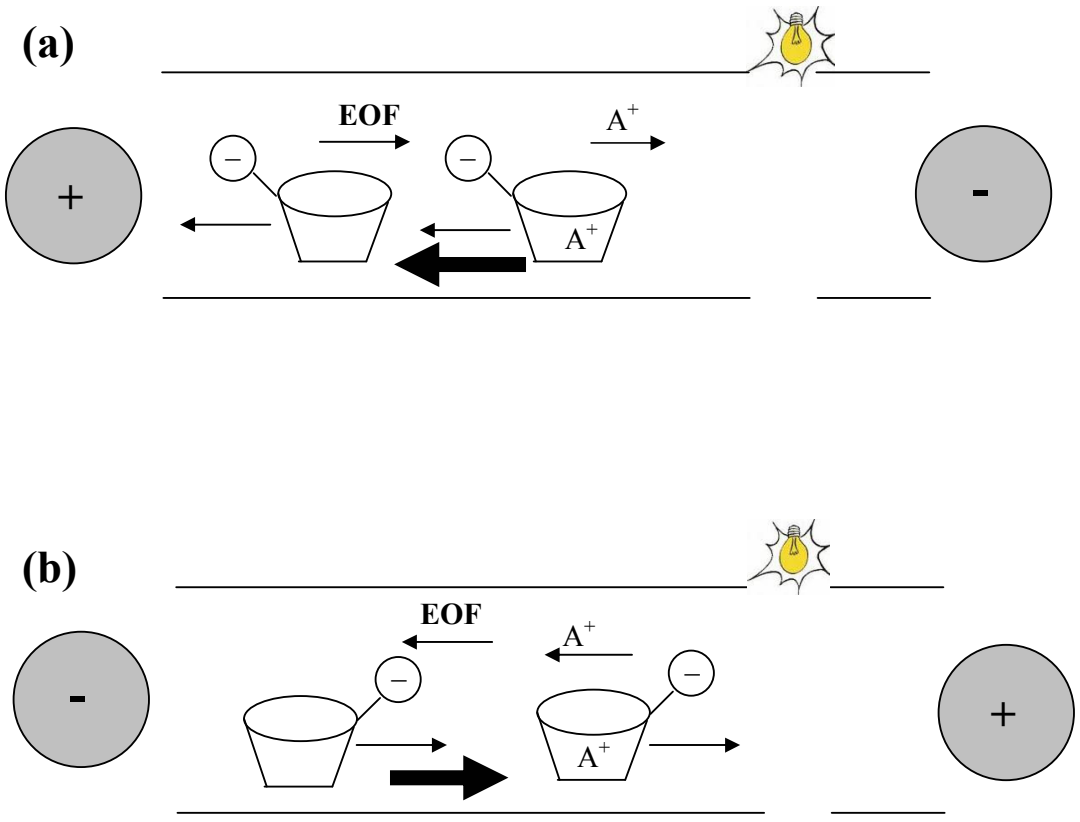


Figure 55: Schematic representation of basic enantiomers separation using acidic phosphate buffer pH 2.5 under normal polarity (a) and reversed polarity (b). (A^+ = ionized basic analyte)

For verapamil HS-β-CD from Sigma gave a resolution R_s value = 7.42 compared to a resolution of R_s value = 1.56 using HS-β-CD from Supelco under the same conditions (Figure 50). For Ephedrine HS-β-CD from Supelco gave a peak with many tops while HS-β-CD from Sigma gave a peak with two tops (Figure 41). Another commercial source difference was noted in HP-β-CD, example is shown in Figure 49 for the separation of verapamil enantiomers were HP-β-CD from Supelco gave a peak with

shoulder while that from Sigma gave only one peak under the same conditions. These differences between the separation tendencies of a certain derivatized cyclodextrin when obtained from different commercial sources could be attributed to difference in derivatization degree so difference in quality. This difference in derivatization degree could also be noted to a lesser extend when different batches from the same commercial company are used were the reproducibility of the separation could also differ.

Changing the type of cyclodextrin not only affects the separation of the two enantiomers but could also invert the migration order when used under the same electrophoretic conditions. Figure 48 (b) and (c) show the separation of L and D tryptophan using either α-CD as in (b) or CM-β-CD as in (c). Under the same separation condition L migrate first when α-CD was used and migrate behind D when CM-β-CD was used. This inversion of migration order could be important for enantiomeric impurity determination, were it is preferably to have the impurity peak in front of the peak of the main enantiomer as they are less likely to overlap and should have better resolution. In contrast when the peak of the impurity migrate behind the peak of the main enantiomer (in the tail of the peak of the main enantiomer) they are more likely to overlap and will probably have a lower resolution value. Whenever the neutral and charged cyclodextrin gave a comparable separation, neutral CD will be selected as it is the cheaper one and has more defined quality.

The best results for the screening of each enantiomeric drug are listed in Table 28 and will be used as a starting point for the optimization process. A 5 m*M* phosphate buffer pH 2.5 (10 folds dilution of the running buffer) has been used as a sample solvent to allow the solubility of the basic enantiomers and provide the required stacking effect of the sample in the capillary. A constant voltage in high of 25 kV was found to provide the separation in a short time with an acceptable heat production.

This screening step is the first step for a three steps project that aims to quantitate enantiomeric impurities. The screening will be followed by optimization step and finally impurity quantitation using the optimized methods. In the screening step all cyclodextrins have been used at a nominal concentration of 2 %. The cyclodextrin that best resolves the enantiomers should be selected for further optimization. When more than one is successful the least expensive cyclodextrin may be the best choice keeping in mind good resolution and reasonable analysis time.

Table 28: List of the cyclodextrins that lead to the best overall separation whether neutral or charged CD under normal or reversed polarity for the ten tested basic enantiomeric drugs.

Basic enantiomeric drug	Best CD for separation	Separation mode	Resolution value (R_s)
atenolol	HS-γ-CD	reversed polarity	R_s = 2.54
alprenolol	HS-γ-CD	reversed polarity	R_s = 1.93
ephedrine	HS-β-CD	normal polarity	peak with shoulder
isoprenaline	HS-β-CD (Sigma)	reversed polarity	R_s = 5.74
methadone	HS-β-CD (Sigma)	normal polarity	R_s = 4.32
pindolol	HS-γ-CD	reversed polarity	two bad shaped peaks
promethazine	HS-α-CD	reversed polarity	peak with two tops
propranolol	CM-β-CD	normal polarity	peak with two tops
tryptophan	α-CD	normal polarity	R_s = 2.45
verapamil	HS-α-CD	reversed polarity	R_s = 10.19

For the drugs where no good baseline separation was obtained with any of the tested seventeen cyclodextrins, an intensive method optimization should be done using the cyclodextrin that gave a beginning of separation (e.g. a peak with shoulder or peak with two tops). The optimization will be done by changing certain conditions in order to improve the separation in term of resolution and analysis time. It is usually divided into primary and secondary optimization. Primary optimization includes the fine-tuning of the CD concentration in the range of 2 - 20% [104, 105], addition of organic modifier to the running buffer (e.g. methanol) [106, 107], changing the buffer pH [108], and the use of a mixture of two CDs in a dual system [109, 110]. Furthermore, various buffer additives as amines and cellulose derivatives can be employed to change the selectivity of the separation by altering electrophoretic mobility [34]. Secondary optimization includes variation of ionic strength to enhance stacking and increasing the voltage to decrease the migration time.

The optimized method can then be used to quantitate enantiomeric impurities. As stated in the ICH guideline Q6A [111], the impurity in the chiral new drug substance should otherwise be treated according to the principles established in the guideline

on impurities in new drug substances. Limits of 0.1% enantiomeric impurity are widely accepted as threshold in the testing of single enantiomer drug substances [112-114]. Thus, enantiomeric separation by CE is important for enantiomeric impurity testing.

The work done so far was for the screening of basic enantiomeric drugs. For acidic compounds neutral or cationic cyclodextrin are usually used. Cationic CDs such as 6-methylamino-β-CD and heptasubstituted methylamino- β-CD have been applied for the separation of acidic and neutral compound [115]. For neutral racemats neutral cyclodextrins are not applicable due to the formation of neutral complexes that do not have electrophoretic motilities. Accordingly the use of charged cyclodextrins (whether cationic or anionic) is necessary for the separation of neutral racemic drugs.

Two different CE instruments have been used during this screening process. A UniCAM CE instrument which requires a capillary with a total length of 62 cm and an effective length (length up to the detector) of 48 cm was used. The effective length of 48 cm means a relatively long migration time until the analytes reaches the detection windows. Of course this is also determined by the migration speed of the analytes which is on the other hand controlled by the EOF and the charge to mass ratio of the analytes. The outlet of this instrument is fixed and so the outlet vial can not be changed automatically between runs. The other instrument PrinCE is a relatively modern instrument which requires a capillary with total length of 85 cm but with an effective length of only 31 cm. This instrument is characterized by the ability to change the outlet during the runs which allow additional facilities as changing the buffer outlet automatically after washing with sodium hydroxide before the run to avoid changing buffer pH or changing the outlet when running a series of different analytes.

5. Summary

5.1 HPLC

Nowadays, fast HPLC methods are indispensably required to improve productivity in pharmaceutical analysis and process analytical technology (PAT). Researchers are trying to develop fast LC methods to reduce analysis time without compromising the quality of the results. A fast LC method refers to a reduction in total analysis time while maintaining the needed resolution, thus offering a greater sample throughput and productivity in the analytical laboratory. A possible tool for converting a standard LC method to a fast LC method is the use of monolithic silica columns. Due to the high permeability of monolithic columns which is provided by the bimodal pore structure; high flow rates can be used with acceptable backpressure. Thus, high velocity isocratic elution is applicable to accelerate up elution of the analyzed compounds and hence reduce the total run time. In monolithic columns, the increase in flow rate does not lead to substantial losses in resolution because of the low mass transfer resistance compared to conventional particle-packed columns. From our studies comparing conventional particle-packed to monolithic silica columns we have concluded that methods for the small drug molecules were successfully transferred. For the relatively larger molecule insulin, the method was not completely transferred from conventional to monolithic column at first go. However, optimization by slightly decreasing the percentage of organic modifier in the mobile phase was sufficient for a good resolution on the monolithic column. Rapid analytical procedures could be obtained when replacing the existing HPLC applications by equivalent ones using monolithic columns instead of conventional particulate columns.

Furthermore, the growing use of such columns requires the definition of a clear strategy for method development. Reversed phase HPLC method development strategies for conventional columns should be updated to meet the additional possibilities of monolithic columns.

The most important advantages of developing methods with monolithic silica columns include the ability to save time for finding initial separation conditions or further optimization of selected conditions. This is due to the applicability of high flow rates and so shorter run and equilibrium times. Monolithic columns have also shown to be advantageous in developing methods for complex mixtures, by connecting two or more columns together to increase the separation efficiency. The longer analysis

time due to columns connection can then re-decreased by applying a proper flow program. Usually, when some compounds of the analyzed mixture have close retention time values, while others have not, loss of resolution between the closely related peaks limits the ability for further reduction in analysis time. In such cases, further reduction in chromatographic run time could be achieved by the application of flow rate programming which is only possible by using monolithic columns.
The use of flow rate gradient (flow-rate programming) in HPLC has an important advantage over gradient elution as system equilibration is not required after each separation. This is important to achieve fast analysis of a series of samples.

Monolithic columns will be one of the most important tools for efficient analysis in the coming years. They will provide excellent and fast analyses with minimal costs using the conventional HPLC systems but with minimal mechanical stress. This new trend will be highly important for the quality control of drugs. It may be applied for processing a large number of samples in a short time, thus being a practical choice for routine quality control studies. Furthermore, the newly introduced 3 mm ID C18 monolithic columns will enable analysts to run samples at a lower flow rates but with similar resolution as the 4.6 mm I.D. monolithic columns. This will provide the ability to directly couple monolithic columns to mass spectrometers without requiring a post column split. Adding the efficiency of monolithic columns to the very high selectivity in mass spectrometry will open new possibilities in important fields like proteome analysis. Many monolithic stationary phases will be developed in the near future that will widen their applicability.

5.2 CE

CE is a useful addition to the separation techniques available for the resolution and quantitation of enantiomers. Particular features of chirally selective CE methods may include simplicity, ruggedness, and low cost when compared to the purchase of expensive chiral selective HPLC columns. In CE, compared to chromatography, a chiral environment can easily be created by filling the capillary with an electrolyte solution containing a chiral selector. However, method development for chiral separations in CE requires experimental and theoretical training.

Initial selections of the best cyclodextrin for the chiral separation of the ten tested basic compounds have been done using a proposed screening strategy and based on the measured resolution. For the tested compounds satisfactory initial enantiomeric separations have been obtained. This approach can also be used to test a broad range of chiral compounds including basic and neutral compounds. It was observed that negatively charged CDs gave better results compared to the neutral CDs. Furthermore, they gave a better enantiomeric separation of the basic drugs when applied under reversed polarity due to stronger interaction with the positively charged analytes. In general, substituted cyclodextrins are in many cases more effective than the native ones in the separation of enantiomers. According to recent works high sulphated cyclodextrins are the most efficient ones. However, whenever possible neutral CDs are generally preferred because of low cost, more defined quality and broad availability.

6. References

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